CHARACTERIZATION OF A YEAST NUCLEOLAR PROTEIN Nop2p

BY

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This work is dedicated to my parents

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Major Department: Anatomy and Cell Biology

The main function of the nucleolus is the synthesis of ribosomes, which involves a series of complicated events, including processing and modification of pre-rRNA. It has been proposed that nucleolar proteins play important roles in these events. However, only a few nucleolar proteins have been identified so far, and the detailed molecular mechanisms related to ribosome synthesis remain elusive. The yeast nucleolar protein Nop2p is essential for cell viability and growth in *Saccharomyces cerevisiae*. In order to dissect its function, conditionally lethal strains were constructed either by placing the open reading frame under the control of a repressible promoter, *GAL10*, or by mutagenizing the gene. Depletion of, and mutation in, Nop2p caused a significant reduction in the levels of the 25S rRNA and the 60S ribosomal subunit. Pulse-chase analysis of the processing of pre-rRNA in these conditionally lethal strains revealed that the processing was significantly delayed with the accumulation of 35S, 32S and 27S pre-rRNAs , and with a significant reduction in the formation of 25S and 5.8S rRNAs. Northern blotting analysis indicated that both 27SA and 27SB pre-rRNAs accumulated during Nop2p depletion. Since protein alignment

analysis suggests that the protein may have methylase activity, the role of Nop2p in methylation of rRNAs was investigated by RNase protection assay. Depletion of Nop2p caused a statistically significant reduction in methylation at a conserved site in 27S pre-rRNA. Mutational analysis of the protein revealed that the multiple missense mutations in the gene were responsible for the temperature-sensitive or formamide-sensitive phenotypes. The mutations in the six *nop2* alleles caused a defect in the processing of pre-rRNA to 25S rRNA. The 27S pre-rRNA, which accumulated in the other four *nop2* alleles, was rapidly degraded in *nop2-4* and *nop2-5* alleles. These findings clearly indicate that Nop2p is required for the synthesis of 60S ribosomal subunit and processing of 27S pre-rRNA to 25S and 5.8S rRNAs. This study also helps elucidate the molecular mechanisms related to ribosome synthesis.

INTRODUCTION

The Nucleolus and Ribosome Synthesis

The nucleolus is a membrane free organelle within the nucleus. Morphologically, three substructures can be recognized in the interphase nucleoli of higher eukaryotic cells: fibrillar centers (FCs), dense fibrillar component (DFC) and granular component (GC). (Warner, 1990; Scheer et al., 1993; Scheer and Weisenberger, 1994). Since FCs contain rDNA and the enzymes essential for its transcription (RNA polymerase I, DNA topoisomerase I and transcription factors), it is believed that the primary rRNA transcript is generated in FCs. Fibrillarin, a nucleolar protein, was found to be localized in this substructure. Since fibrillarin is involved in pre-rRNA processing, DFC is likely the site of early steps in pre-rRNA processing. The late events of maturation of ribosome precursor particles occur in GC. The yeast nucleolus appears as a single, crescent-shaped region occupying more than 30% of the nucleus (Woolford, 1991). Although there are no obviously distinct substructures in the yeast nucleolus, ribosome synthesis is also the main function of yeast nucleolus (Warner, 1989; Melese and Xue, 1995).

The yeast ribosome is a large complex of proteins and RNAs, which consists of 40S and 60S ribosomal subunits. The 40S subunit contains about 32 different proteins and an 18S rRNA, while the 60S subunit contains about 45 proteins and three rRNAs: 5S, 5.8S and 25S (28S in higher eukaryotes) rRNAs (Woolford, 1991). Since the ribosome is a protein-synthesizing machine, synthesis of the ribosome itself is essential for cell growth and maintenance. The nucleolus is the center for ribosome synthesis (Table 1). In the nucleolus, repeated rDNA genes are transcribed by RNA polymerase I into a single large

Table 1. Main Events in Ribosome Synthesis

Event	Location	Comments
rDNA transcription	Nucleolus	Transcribed by RNA polymerase I into a large single precursor containing 18S, 5.8S and 25S rRNA sequences.
5S rDNA transcription	Nucleolus	Transcribed by RNA polymerase III; no processing or modification has been found.
Pre-rRNA processing	Nucleolus (except 20S to 18S rRNA in the cytoplasm)	Involving a series of cleavages; simultaneous with the modification and assembly.
Modification of rRNAs	Nucleolus	Including base methylation, ribose methylation, and pseudouridylation during the transcription.
Transcription of ribosomal protein (r-protein) genes	Nucleus	RNA polymerase II.
Processing of mRNAs of r-proteins	Nucleus	Including capping, splicing and polyadenylation.
Translation of r-proteins	Cytoplasm	
Modification of r-proteins	Cytoplasm or nucleolus?	Including methylation, phosphorylation and acetylation.
Import of r-proteins	Cytoplasm to nucleolus	Involving nuclear localization signal (NLS) or "piggy-back import" mechanism.
Assembly	Nucleolus	Involving protein-protein and protein-RNA interaction.
Export of ribosome subunits	Nucleolus to cytoplasm	

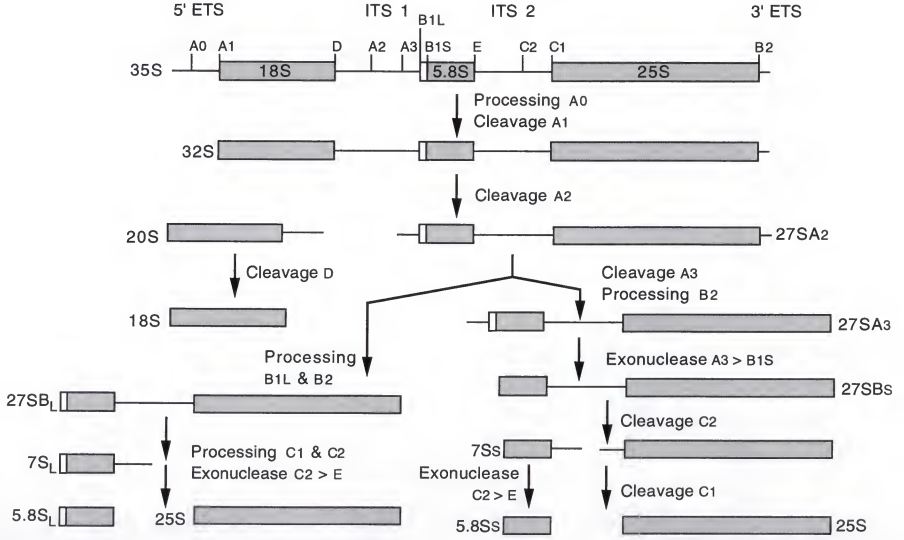
35S (45S in higher eukaryotes) precursor, which is then modified and cleaved into three mature rRNAs: 18S, 5.8S and 25S rRNAs. 5S rRNA is generated by RNA polymerase III. Coupled with this maturation process, ribosomal proteins, imported from the cytoplasm, are assembled with pre-rRNAs and 5S rRNA to form functional 40S and 60S ribosomal subunits. The ribosomal subunits are then exported to the cytoplasm for protein synthesis (Warner, 1989; Woolford and Warner, 1991; Sollner-Webb et al., 1996).

Processing of Pre-rRNA

The single, large pre-rRNA contains 18S, 5.8S and 25S rRNA sequences, which are flanked by 5' and 3' external transcribed spacer (ETS), and separated by internal transcribed spacer (ITS) 1 and 2 (Figure 1). Release of the rRNAs from the precursor is, therefore, essential for ribosome synthesis. Processing of pre-rRNA into mature rRNAs involves multiple endonucleolytic and exonucleolytic cleavages. Earlier studies of pre-rRNA processing in unicellular organisms (*Saccharomyces cerevisiae*), plants (*Phaseolus vulgaris*), insects (*Drosophila*), amphibia (*Xenopus laevis*) and several mammals suggest that a common processing pathway is generally followed (Crouch, 1984; Hadjiolov, 1985; Tollervey, 1987; Gerbi et al., 1990; Eichler and Craig, 1994; Sollner-Webb et al., 1996).

In yeast, the processing starts at 5' ETS (Figure 1). Cleavage at A_0 generates a 33S intermediate, which is then converted to 32S with the processing at A_1 site. These early processing steps generate the 5' end of 18S rRNA. Cleavage at A_2 within ITS1 separates the processing pathway into two halves. One is conversion of 20S into 18S rRNA, which occurs in the cytoplasm. Another is to form 5.8S and 25S rRNAs. When the processing of ITS1 and 3' ETS is completed, 27SA becomes 27SB. 27SB is the common precursor for 5.8S and 25S rRNAs. Cleavage at C_2 site within ITS2 generates two intermediates. One contains ITS2 and 25S rRNA sequence. With the subsequent processing at C_1, the mature 25S rRNA is formed. Another is 7S intermediate, the direct precursor for 5.8S

Figure 1. The pre-rRNA processing pathway in *Saccharomyces cerevisiae*. The rDNA is transcribed into a single 35S pre-rRNA, which contains the 5' external transcribed spacer (ETS), the sequences of 18S, 5.8S and 25S rRNAs separated by the internal transcribed spacer (ITS) 1 and 2, and 3' ETS. To release the rRNA sequences, the 35S pre-rRNA undergoes a series of endonucleolytic and exonucleolytic cleavage steps as indicated. The cleavage at A_2 site in ITS1 separates processing into the two pathways leading to the rRNAs of the large and small ribosomal subunits, respectively. The processing of 20S to 18S rRNA occurs in the cytoplasm. The cleavage of 27SB at C_2 site in ITS2 generates the precursors for 5.8S and 25S rRNAs, respectively. There are two forms of 5.8S rRNA: the major short form $5.8S_S$, and the minor large form $5.8S_L$ (with the extra 7-8 nucleotides at the 5' end, Henry et al., 1994).


5' ETS
ITS 1
B1L
ITS 2
3' ETS
A0 A1
D
A2
A3
B1S
E
C2
C1
B2
35S
18S
5.8S
25S
Processing A0
Cleavage A1
32S
Cleavage A2
20S
27SA2
Cleavage D
18S
Cleavage A3
Processing B2
27SA3
Processing
B1L & B2
Exonuclease A3 > B1S
27SBL
27SBS
Cleavage C2
7SL
Processing C1 & C2
Exonuclease C2 > E
7SS
Exonuclease
C2 > E
Cleavage C1
5.8SL
25S
5.8SS
25S

rRNA. With exonucleolytic "chewing" from C_2 to E site, 7S is converted to 5.8S rRNA (Tollervey, 1987). A recent study revealed that there are two versions of 5.8S rRNA in the wild type cells. The larger 5.8S ($5.8S_L$) containing several extra nucleotides at 5' end is a minor version (Henry et al., 1994).

Modification of rRNAs and Ribosomal Proteins

Methylation of rRNAs

All eukaryotic rRNAs are modified by methylation, though the number of the methylations varies among different organisms. There are a total of 116 methylations in human rRNAs, while only 65 methylations in yeast rRNAs (Maden et al., 1995). Nevertheless, from yeast to human, a great majority (80-90%) of the methylations are 2'-O-methylations. No consensus sequence for rRNA methylation has been found. However, almost all eukaryotic rRNA methylations are in the conserved core regions of rRNA: the regions with main secondary structure features conserved from archaea to bacteria to eukaryotes (Maden, 1990; Maden, 1996). In yeast, most methyl groups are added soon after transcription at the level of 35S and 32S precursors (early methylation). Very few are added at the level of the late processing intermediates at the level of 27S and 20S intermediates (late methylation). Among the methylations identified in the 25S rRNA sequence, only four were found to be added at the level of 27S level: two copies of mU (base methylations) and UmGmΨUC2922 (2'-O-methylations) (Brand et al., 1977).

The function of the methylation remains elusive. Since methyl groups add hydrophobic patches to rRNA and since all methylations are in the conserved core regions, the modification may facilitate rRNA folding or rRNA-protein interactions, and thus may be involved in ribosome synthesis. Some earlier studies found that the methylation of rRNAs was required for pre-rRNA processing and maturation of ribosomes. In cultured

mammalian cells, methionine deprivation or treatment with ethionine, a chemical inhibitor of methyl transfer, caused a defect in ribosome maturation and pre-rRNA processing (Swann et al., 1975; Caboche and Bachellerie, 1977; Wolf and Schlessinger, 1977).

The methylation of rRNA may also be involved in the ribosome function. A higher concentration of methyl groups was found in and around the peptidyl transfer region of the large subunit rRNA, and several modifications in 18S rRNA were identified on the platform of the small ribosomal subunit, where mRNA and tRNA binding occurs (Maden, 1995). Demethylation of rRNAs was found to be related to the reduced protein synthesis (Wolf and Schlessinger, 1977). Recent studies of Dim1p (a yeast 18S rRNA methylase) and a yeast temperature-sensitive *nop1* mutant suggest that the ribosome containing the demethylated rRNAs was defective in its function. However, the demethylated pre-rRNA was still processed and methyl deficient ribosome could still be formed (Tollervey et al., 1993; Lafontaine et al., 1995).

These findings suggest that rRNA methylation may be required for ribosome function. Nevertheless, the relationship between methylation and pre-rRNA processing and ribosome maturation has not been clearly understood.

Pseudouridylation of rRNAs

Eukaryotic rRNAs are also modified by pseudouridylation. The ratio of pseudouridylation to 2'-O-methylations is close to 1:1 in yeast (47:55), amphibian (98:98) and human (95:105) rRNAs (Maden et al., 1995), suggesting that the two types of modifications may be interdependent. Since pseudouridine has more versatile hydrogen-bonding properties than uridine, it was speculated that pseudouridine contributes to the recognition events crucial to ribosomal function. However, the exact function of this modification has not been understood.

Modification of Ribosomal Proteins

Ribosomal proteins were found to be modified by methylation, phosphorylation or acetylation (Raue and Planta, 1991; Woolford and Warner, 1991). Several yeast ribosomal proteins belonging to the small subunit (S31 and S2) and to the large subunit (L3, L15, L41, and L42) were found to be methylated (Cannon et al., 1977; Kruiswijk et al., 1978). Since addition of a methyl group increases the local hydrophobicity, it is possible that methylation of ribosomal proteins may be related to protein-protein association during the assembly. The study of methylation of the ribosomal protein L3 in *E.coli* supports this possibility. The only modification in L3 is N^5-methylglutamine, which is modified by the prmB protein. The *E.coli* mutant lacking this methylase activity displayed a cold-sensitive phenotype. At 22°C, the mutant cells synthesize abnormal ribosomal particles that are very unstable and quickly degraded (Lhoest and Colson, 1977; Muranova et al., 1978; Lhoest and Colson, 1981). However, in eukaryotic cells, such protein methylases have not been identified. The role of methylation of ribosomal proteins in ribosome synthesis is not known.

The function of phosphorylation and acetylation of ribosomal proteins is not known. The lack of acetylation (such as in rp39, rp52, rp56 and rp59) or the lack of phosphorylation (such as in S10) did not affect cell growth, suggesting that these modifications may not function in ribosome synthesis (Johnson and Warner, 1987; Woolford and Warner, 1991; Wachtler and Stahl, 1993).

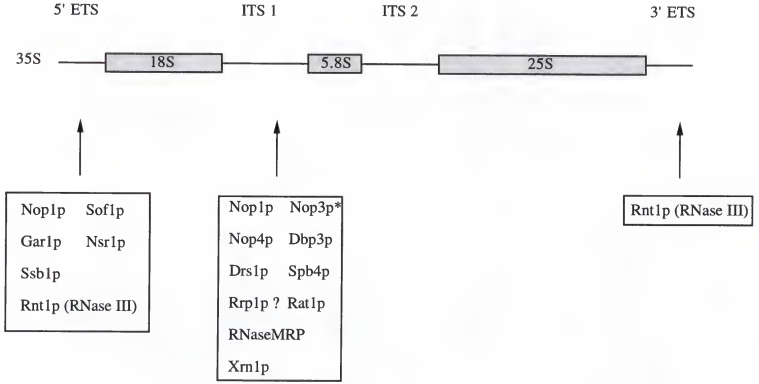
Functions of Nucleolar Proteins

Pre-rRNA Processing

Non-ribosomal proteins in the nucleolus are thought to play important roles in the synthesis of ribosomes. The number of these nucleolar proteins is believed to be very large (Hernandez-Verdun, 1991). A number of the nucleolar proteins required for rDNA transcription have been identified (Reeder, 1990). However, the functions of the nucleolar proteins in the other events of ribosome synthesis have just recently become understood. Although the general outline for pre-rRNA processing (Figure 1) has been known for years, the enzymes and regulatory factors required for the processing have been largely unknown until recently. Several nucleolar proteins, such as fibrillarin, B23 and nucleolin, were described in high eukaryotic cells years ago (Michalik et al., 1981; Ochs et al., 1985; Caizergues et al., 1987). However, their functions remain unclear, mainly because of technical difficulties in the study of high eukaryotic cells. The yeast system is easily accessible to powerful genetic and molecular biology techniques. Recent studies in the yeast model have generated a large amount of knowledge about the roles of the nucleolar proteins in pre-rRNA processing (Figure 2).

Fibrillarin, localized in the DFC of the nucleolus, was first found in *Physarum* and later identified in mammalian cells (Christensen et al., 1977; Ochs et al., 1985). Since its yeast homologue, the Nop1 protein (Nop1p), was identified (Aris and Blobel, 1991), the protein function has been extensively studied. Human or *Xenopus* fibrillarin can complement a yeast *nop1* mutant (Jansen et al., 1991), suggesting that the protein function is evolutionarily conserved. By genetic manipulation, *NOP1* was placed under the control of a repressible promoter. Depletion of this protein caused a progressive impairment of all pre-rRNA processing steps, particularly the pathway leading to 18S rRNA (Tollervey et al., 1991). The finding suggests that Nop1p is required for the processing in 5' ETS and

Figure 2. Yeast nucleolar proteins implicated in the processing of pre-rRNA. The nucleolar proteins listed in the boxes are required for the processing of pre-rRNA. Depletion of, or mutation in, the proteins separately caused a reduction in the production of rRNAs. Some of the proteins are involved in the processing in more than one spacer region. The arrows indicate the spacer regions of 35S pre-rRNA requiring the nucleolar proteins for processing. *: Nop3p is localized in the nucleus.


5' ETS
ITS 1
ITS 2
3' ETS
35S
18S
5.8S
25S
Nop1p Sof1p
Gar1p Nsr1p
Ssb1p
Rnt1p (RNase III)
Nop1p Nop3p*
Nop4p Dbp3p
Drs1p Spb4p
Rrp1p ? Rat1p
RNaseMRP
Xrn1p
Rnt1p (RNase III)

ITS1. Further study of the temperature sensitive (ts) mutations in *NOP1* gene showed that the five *nop1* ts alleles affected ribosome synthesis at different steps, including pre-rRNA processing, pre-rRNA modification and ribosome assembly (Tollervey et al., 1993). The Sof1 protein was isolated by screening for the extragenic suppressors of the ts phenotype caused by incomplete complementation of a *nop1* disruption by human fibrillarin. Sof1p was found to be associated with Nop1p, and depletion of it caused a defect in 18S rRNA production (Jansen et al., 1993), suggesting that Sof1p is required for processing in the 5' ETS and/or ITS1. Another nucleolar protein required for 18S rRNA formation is Gar1p, which shares a glycine, arginine-rich domain (GAR domain) with Nop1p (Girard et al., 1992). Nop3p, also sharing a GAR domain with Nop1p, is required for the conversion of 27SB pre-rRNA to 25S rRNA, and 20S pre-rRNA to 18S rRNA (Russell and Tollervey, 1992). Nop77p was isolated by screening for genes synthetically lethal with *nop1-5* ts allele (Berges et al., 1994). The same protein was also identified by another lab at the same time and termed Nop4p (Sun and Woolford, 1994). Nop77p/Nop4p is required for the conversion of 27SA to 27S B pre-rRNA, since no 27SB pre-rRNA was formed in the cells depleted of the protein. This suggests that the protein is required for the processing in 3' ITS1. All these findings indicate that fibrillarin (Nop1p) is the center of more than one protein-protein and protein-snoRNAs (see below) complex, which mainly functions in the processing of 5' ETS and ITS1.

Several putative ATP-dependent RNA helicases in yeast have been characterized. These proteins contain the evolutionarily conserved DEAD-box. The *DRS1* gene was isolated by complementation of cold-sensitive phenotype of *drs1*. In the *drs1* mutant, processing of 27S pre-rRNA to 25S rRNA was impaired (Ripmaster et al., 1992). Another yeast putative RNA helicase is Spb4p, which is also involved in the processing of 27S pre-rRNA to 25S rRNA (Sachs and Davis, 1990). However, it is not clear if Drs1p and Spb4p are involved at the step of 27SA to 27SB pre-rRNA, or at the step of 27SB to 25S rRNA. Recently, another putative RNA helicase, Dbp3p, was isolated. This protein

was found to be required for processing at A_3 site in ITS1. The gene is not essential. However, disruption of it caused a delay in the processing of 27SA to 27SB pre-rRNA, and thus a significant reduction in the formation of 25S rRNA (Weaver et al., 1997). The mechanism by which these putative RNA helicases are involved in pre-rRNA processing is not clear. It was speculated that RNA helicase may modulate the secondary structure of pre-rRNA for recruiting the RNase MRP complex (see below) to A_3 site (Weaver et al., 1997).

Several other proteins were found to be involved in processing ITS1. In *rrp1* ts mutant, 27S pre-rRNA was rapidly degraded and the formation of 25S rRNA was reduced (Fabian and Hopper, 1987). It is not clear if 27SB pre-rRNA was formed or not. RNase MRP, an endoribonuclease, is required for processing at A_3 site in ITS1. Mutation in its protein component (ts *pop1-1*) or in its RNA component (*rrp2-1*) caused a defect in the cleavage at the A_3 site (Lygerou et al., 1994; Morrissey and Tollervey, 1995). Xrn1p and Rat1p were found to have 5' to 3' exonuclease activity. Mutation in *RAT1* gene caused a reduction in the formation of the short form of yeast 5.8S rRNA (Amberg et al., 1992). Mutation in *XRN1* gene resulted in the accumulation of the excised 5' region of ITS1, between the 3 end of 18S rRNA and A_2 site (Stevens et al., 1991). Rat1p and Xrn1p were found to digest from site A_3 to B_1 (s) to form the 5' end of 27SB (s) pre-rRNA (Henry et al., 1994).

Yeast *RNT1*, a homologue of bacterial RNase III, was found to encode a double-strand endoribonuclease, which is required for processing both the 5' and the 3' ETS. This endonuclease activity was reproduced *in vitro* in the absence of snoRNA, suggesting that snoRNA had no catalytic role in this situation (Elela et al., 1996). Nsr1p, a yeast homologue of nucleolin, is required to form 20S pre-rRNA, a direct precursor for 18S rRNA. The cells lacking *NSR1* were viable but had a severe growth defect and an near absence of 20S pre-rRNA (Lee et al., 1992).

Since pre-rRNA processing involves multiple orderly cleavage steps (Figure 1), it is not surprising that so many nucleolar proteins are required for its processing. So far, no yeast nucleolar protein has been clearly shown to be required for ITS2 processing. The detailed mechanisms for the other processing steps have still not been completely understood. Therefore, much more remains to be learned about the functions of the nucleolar proteins in pre-rRNA processing.

Modification of rRNAs

It has been known for many years that all eukaryotic rRNAs are modified by methylation (Maden, 1990). However, only recently have several putative eukaryotic rRNA methylases and the regulatory factors been characterized. Nop1p was found to be required for rRNA methylation (Tollervey et al., 1993). Since Nop1p is associated with a large number of the nucleolar proteins and snoRNAs (see below) and since it was found to be involved in every major step of ribosome synthesis (Tollervey et al., 1993), it is most possible the Nop1p is a multi-functional snoRNP protein. Human proliferation-associated nucleolar protein p120 was found to contain a sequence conforming to an S-adenosyl-methionine (SAM) binding domain, which is present in a variety of the known methylases (Koonin, 1994). However, it has not been shown that p120 possesses rRNA methylase activity. Segal and Eichler identified and partially purified a novel nucleolar methylase from the crude mouse nucleolar extracts. This protein was found to methylate the 28S rRNA transcript at a unique site *in vitro* (Segal and Eichler, 1991). However, the gene has not been isolated.

Recently, two yeast rRNA methylases have been identified. Pet56 protein was found to be required for ribose methylation at a universally conserved nucleotide in the peptidyl transferase center of the mitochondrial large ribosomal RNA (21S rRNA). This was confirmed by the result from the *in vitro* methylation reaction. The reduction in this

protein function caused a defect in the formation of large subunit of mitochondrial ribosome (Sirum-Connolly and Mason, 1993), which suggests that the rRNA methylation may be required for ribosome assembly or stability. Yeast Dim1 protein was cloned by complementation of the kasugamycin sensitivity of the *E.coli ksg* A mutant (Lafontaine et al., 1994). KsgA is a 16S rRNA dimethylase in *E.coli* (van Buul and van Knippenberg, 1985). The study of Dim1p function showed that this protein also had dimethylase activity in yeast. This protein was found to be required for the methylation at a specific site on 18S rRNA. However, such methylation was not required for the formation of 18S rRNA, though Dim1p itself was essential for cell viability (Lafontaine et al., 1995).

Considering that all eukaryotic rRNA are methylated at a large number of sites, more rRNA methylases probably remain to be identified, and the significance of rRNA methylation in ribosome synthesis remains to be studied.

Modification of Proteins

Ribosomal proteins are covalently modified (Raue and Planta, 1991; Woolford and Warner, 1991). However, it is not known if such modification occurs in the cytoplasm and/or the nucleolus. Since all of the ribosomal proteins are imported to the nucleolus to assemble with rRNAs into ribosome subunits, it is possible that the nucleolar proteins may modify some of the ribosomal proteins to facilitate the assembly.

Some nucleolar proteins, such as nucleolin, fibrillarin, and Ssb1p, were found to be methylated (Lischwe, 1990). All of these nucleolar proteins contain the glycine, arginine-rich domain (GAR domain) interspersed with phenylalanine, which is thought to be recognized by a group of protein methylases (Lischwe, 1990). The methyl groups were found to be added to the arginine residues as N^G, N^G-dimethylarginine (Lischwe et al., 1982; Lischwe et al., 1985). The exact function of the arginine methylation is not known. Since the GAR domain was found to have a high affinity for nucleic acid (Cobianchi et al.,

1988), it was believed that such modification is related to nucleic acid binding. Since the methyl group increases the local hydrophobicity, a role of the modification in protein-protein interaction is also possible (Lischwe, 1990). However, the methylases required for the modification have not been identified. These nucleolar proteins are mainly localized in the nucleolus (Lischwe et al., 1985; Lapeyre et al., 1987; Clark et al., 1990), Therefore, it is possible that other nucleolar proteins with protein methylase activity may modify these proteins.

Assembly of Ribosomes

Ribosome assembly involves association of approximately 70~80 ribosomal proteins and 4 rRNAs through protein-protein and protein-rRNA interactions. In *E. coli*, ribosomal subunits can be successfully assembled *in vitro* with only rRNA and ribosomal proteins (Nomura, 1973). However, such *in vitro* assembly has not been successful for eukaryotic ribosomes, suggesting that some cofactors are required for proper assembly. *In vivo* labeling in yeast showed that ribosomal precursor particles contained not only ribosomal proteins, but also a number of non-ribosomal proteins (Warner, 1971), suggesting that nucleolar proteins may participate in the assembly process.

Nucleolin and B23 in higher eukaryotes were found to be associated with pre-ribosomal particles in the granular component of the nucleolus (Spector et al., 1984; Biggiogera et al., 1989), where pre-ribosomal subunits occur. Since both proteins contain acidic domains (Spector et al., 1984; Lapeyre et al., 1987), it was speculated that the proteins may function by facilitating assembly of the many basic ribosomal proteins with rRNAs (Woolford, 1991). Several other nucleolar proteins also contain an acidic domain, including Nopp140, Nsr1p, Drs1p, and Nop77p/Nop4p (Melese and Xue, 1995). It was believed that the acidic domain in these proteins may interact with the highly basic NLS or NLS-like sequences in many ribosomal proteins and thus to facilitate ribosome assembly

(Xue and Melese, 1994; Melese and Xue, 1995). The glycine, arginine-rich domain (GAR domain) is present in many nucleolar proteins, including fibrillarin (Nop1p), Nsr1p, Ssb1p, nucleolin, Gar1p, and Nop3p (Schimmang et al., 1989; Lee et al., 1991; Jong et al., 1987; Lapeyre et al., 1987; Girard et al., 1992; Russell and Tollervey, 1992). It is possible that GAR domain in these proteins may promote proper assembly of ribosome by interacting with some acidic ribosomal proteins, such as yeast A0, A1, L44, L44' and L45 (Woolford and Warner, 1991).

Since ribosome assembly involves interaction among ribosomal proteins and rRNAs, modification of rRNA by the nucleolar proteins may play a role in assembly. One piece of evidence is from the study of a yeast ts *nop1.3* mutant (Tollervey et al., 1993). In this mutant, the methylation of pre-rRNA was strongly inhibited at 36°C, but the demethylated pre-rRNA was still processed. However, the lethal phenotype caused by this allele at 36°C suggests that the synthesized ribosomes were non-functional.

Functions of SnoRNAs

Small nucleolar RNAs (snoRNAs) have been identified in a variety of eukaryotic cells (Fournier and Maxwell, 1993). The number of snoRNAs is very large. In yeast, more than 30 snoRNAs have been isolated (Balakin et al., 1996). Some snoRNAs have been shown to be required for pre-rRNA processing. Depletion of either U3, U14, or snR30 caused a severe defect in the formation of 18S rRNA (Li et al., 1990; Hughes and Ares, 1991; Morrissey and Tollervey, 1993), suggesting that these snoRNAs function in the processing of 5' ETS and/or ITS1 (Fournier and Maxwell, 1993). SnR10, though not essential, participates in the processing of 35S pre-rRNA (Tollervey, 1987). However, other snoRNAs have not been found to be essential for cell viability and pre-rRNA processing (Sollner-Webb et al., 1996).

Recently, it has been found that a family of snoRNAs are required for the site selection of rRNA 2'-O-methylation (Maden, 1996; Tollervey, 1996). There are two important features about these snoRNA. First, they contain two evolutionarily conserved motifs, boxes C and D, which are believed to be protein binding sites. Second, the snoRNAs exhibit long (14-17 nucleotide) regions of perfect complementarity to pre-rRNA (Maden, 1996; Tollervey, 1996). Mutations altering the sequence of the snoRNA or the spacing between box D and the region of base paring is sufficient to change the specificity of methylation (Cavaille et al., 1996; Kiss-Laszlo et al., 1996). These studies suggest that the 2'-O-methyltransferase may consist of a protein component, which catalyzes transfer of a methyl group, and an snoRNA component, which selects the modification site.

Interaction of Nucleolar Proteins and SnoRNAs

Several pieces of evidence indicate that nucleolar proteins and snoRNAs may functionally and physically interact. First, depletion of Nop1p, Gar1p, Sof1p and Nsr1p cause phenotypes that are strikingly similar to phenotypes caused by depletion of U3, U14, and snR30, which suggests that these proteins and RNAs function at the same or similar steps of pre-rRNA processing (Li et al., 1990; Hughes and Ares, 1991; Tollervey et al., 1991; Girard et al., 1992; Lee et al., 1992; Jansen et al., 1993; Morrissey and Tollervey, 1993). Second, immunoprecipitation studies indicate that the nucleolar proteins are physically associated with snoRNAs. At least 11 yeast snoRNAs can be precipitated by antibodies to Nop1p (Schimmang et al., 1989). Gar1p and Sof1p were found to be associated with snR10 and snR30 (Girard et al., 1992), and with U3 (Jansen et al., 1993), respectively. Finally, a majority of the nucleolar proteins identified so far (such as Nop1p, Nop3p, Gar1p, Nsr1p, Ssb1p, fibrillarin and nucleolin) contain the consensus motifs of RNA recognition motif (RRM) or/and GAR domain (Ochs et al., 1985; Jong et al., 1987; Lapeyre et al., 1987; Schimmang et al., 1989; Lee et al., 1991; Girard et al., 1992; Russell

and Tollervey, 1992). The RRM consists of an amino acid sequence of ~80 residues conserved among the members of a family of RNA-binding proteins (Kenan et al., 1991). The aromatic and basic residues in the RRM have been implicated in nucleic acid interaction (Kenan et al., 1991). The GAR domain consists of repeating units of RGGFRGG (Russell and Tollervey, 1992). Study of the GAR domain of vertebrate nucleolin suggests that the domain is involved in non-specific protein-RNA association (Ghisolfi et al., 1992). On the other hand, all fibrillarin (Nop1p)-associated snoRNAs contain two consensus sequences, boxes C and D, which may be the binding sites for common proteins (Sollner-Webb et al., 1996). Another family of snoRNAs have consensus ACA triplet positioned 3 nucleotides before the 3' end of the RNA. All members of the yeast ACA family are associated with Gar1p (Balakin et al., 1996). Based on the above evidence, it was proposed that nucleolar proteins are associated with snoRNAs to form snoRNP complex, which may be involved in the processing and modification of pre-rRNA, ribosome assembly and transport (Fournier and Maxwell, 1993).

Nop2p is a yeast homologue of human nucleolar protein p120, which is a marker for cell proliferation (de Beus et al., 1994; Fonagy et al., 1993). Nop2p is different from most of the known nucleolar proteins in that it does not contain either RRM or GAR domain (de Beus et al., 1994). However, like p120, Nop2p contains a putative S-adenosyl-methionine (SAM) binding domain, which is conserved among methylases. In this study, by genetic manipulation, the conditionally lethal strains were constructed and a deficit in Nop2p function was investigated at the restrictive conditions. The relationship between Nop2p and methylation was analyzed. By using immunological and genetic approaches, possible functional interaction between Nop2p and other macromolecules was explored.

METHODS AND MATERIALS

Strains, Media, and Microbiological Methods

Saccharomyces cerevisiae strains used in this study are listed in Table 2. Yeast cells were grown at 30°C or as indicated. The complex media were either YPD (1% yeast extract, 2% peptone, and 2% dextrose) or YPGal (1% yeast extract, 2% peptone, and 2% galactose). The synthetic minimal media were either SD (0.67% yeast nitrogen base and 2% dextrose) or SGal (0.67% yeast nitrogen base and 2% galactose), supplemented with appropriate nutrients (Sherman, 1991). Methionine was always omitted from the synthetic media in this study. For [^{3}H]uracil labeling experiment, the concentration of uracil in the synthetic media was reduced to one third the normal amount (Sherman, 1991). For plasmid shuffling, 1 mg/ml of 5-fluoroorotic acid (5-FOA) (PCR Incorporated) was added to either SD or SGal medium (Boeke et al., 1987). For screening for formamide-sensitive or ethanol-sensitive strains, 3% formamide (Aguilera, 1994) or 6% ethanol (Aguilera and Benitez, 1986) was added into SD medium, respectively. YPD containing low phosphorus (LP-YPD) was prepared as described previously (Reid and Schatz, 1982). Briefly, YP (1% yeast extract, 2% peptone) was treated with 10 mM $MgSO_4$ and 1% NH_4OH at room temperature (RT, ~25°C) for 30 minutes, and filtered twice through Whatman 3MM paper before adding 2% dextrose. For screening for thermosensitive strains, yeast cells were replica plated and grown at permissive temperature (25°C) and non-permissive temperature (14°C or 37°C).

Yeast genetic manipulation was done according to previously described methods (Guthrie and Fink, 1991) with minor changes. Yeast cells were first grown at 30°C on

selective SD plates for 2-4 days. A small dab of cells was patched onto the pre-sporulation plate (0.8% yeast extract, 0.3% peptone, 10% dextrose, and 1.5% agar.) and grown at 30°C for ~24 hours. Cells from pre-sporulation were then made a patch onto the sporulation plate (1% KOAc, 0.1% yeast extract, 0.05% dextrose, 1.5% agar, and with one fourth the normal amount of appropriate nutrients). The spore wall of asci was digested with 6% glusulase (DuPont) at room temperature for 6 minutes. The digested asci were then spread onto a YPD plate, and spores were separated by micromanipulation. The spores were germinated and grown on YPD plates.

DNA Methods

All of the plasmids used in this study (Table 3) were maintained and amplified in *E. coli* DH5α in LB medium (0.5% Bacto-yeast extract, 1% Bacto-tryptone, 0.5% NaCl, pH 7.5, and 1.5% agar for plates only) plus 100 μg/ml ampicillin. Plasmids were introduced into *E. coli* DH5α by using the RbCl method (Hanahan, 1983).

DNA was transformed into yeast cells by using the lithium acetate method (Ito et al., 1983).

Yeast genomic DNA was prepared as described (Wack et al., 1994). An overnight culture of yeast in 2.5 ml of YPD (~5 OD_{600} units) was collected and resuspended in TE buffer (10 mM Tris·HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0) with 0.5 M sorbitol. Cell wall was digested with ~400 units of Lyticase (Sigma) and 5 μM dithiothreitol (DTT) at RT for 30 minutes. Spheroplasts were broken in 200 μl of 50 mM Tris·HCl (pH 8.0), 20 mM EDTA and 1.25% sodium dodecyl sulfate (SDS) at 65°C for 20 minutes. After adding 100 μl of 3 M potassium acetate (pH 5.0), the mixture was centrifuged at top speed. DNA in the supernatant was precipitated with ethanol. RNA in the pellet was eliminated by enzymatic digestion with 2 μg/ml RNase A. DNA was again precipitated by ethanol.

Recovery of plasmid from yeast was done according to the method of Strathern and Higgins (Strathern and Higgins, 1991). Briefly, an overnight culture in 2 mls of YPD was collected and resuspended in 100 µl of TENS buffer (100 mM NaCl, 10 mM Tris·HCl, pH 8.0, 1 mM EDTA, and 0.1% SDS). The cells were broken by vortexing with 0.25 g of glass beads for one minute. The cell lysate was treated with phenol. The crude preparation containing plasmid DNA was obtained by treating the aqueous layer with a Geneclean kit (Bio 101). Using a gene pulser (BioRad), the recovered plasmid DNA (in 3 µl) was introduced into *E. coli.* DH5α.

Electro-competent *E. Coli.* DH5α cells were prepared as described below. An overnight culture of *E. coli.* DH5α in 5 ml of 2X YT medium (1.6% Bacto-tryptone, 1% Bacto-yeast extracts and 0.5% NaCl) was diluted into 500 ml of 2X YT and grown at 37°C for 2-3 hours. The cells were collected and washed twice with ice cold water. The cells were washed with ice cold 10% glycerol. Finally, the cells were resuspended in 1 ml of ice cold 10% glycerol. Aliquots of the cells were stored at -80°C.

The restriction enzymes used in this study were purchased from New England Biolabs. Restriction digestions were performed according to the manufacturer's instructions.

DNA fragments were isolated as described below. After separation of DNA fragments on 0.8% agarose gels, a piece of Whatman GF/C filter paper on a piece of dialysis tubing membrane was inserted facing the band of interest. After electrophoresis for 20-30 minutes, the GF/C paper was removed, and the DNA on the paper was obtained by brief centrifugation. After adding an equal volume of phenol, the mixture was added onto a sephadex G-50 spin column. The DNA was recovered by spinning the column in IEC centrifuge at 1,000 rpm (~250*g*) for 10 minutes.

Southern blotting was done as described (Ausubel et al., 1993). Briefly, after restriction digestion, the DNA samples were separated on a 0.8% agarose gel. The gel was treated sequentially with 0.25 M HCl for depurination, with denaturation buffer (1.5 M

NaCl, 0.5 M NaOH), and with neutralization buffer (3 M NaCl, 0.5 M Tris, pH7.0). Transfer of DNA from the gel to a piece of nitrocellulose paper was done with upward capillary transfer in 20X SSC buffer (3 M NaCl, 0.3 M $Na_3Citrate{\cdot}2H_2O$, pH 7.0). The nitrocellulose paper was then baked at 70°C for 2 hours. Probes were labeled with [α-^{32}P]dCTP by random oligonucleotide priming.

Manual DNA sequencing was done by using the dideoxy method (Ausubel et al., 1993). The double-strand template carried on a plasmid was denatured in 1 M NaOH at 37°C for 5 minutes. The DNA with 2.0 pmol of a primer in 5X sequence reaction buffer (200 mM Tris·HCl, pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl) was incubated at 65°C for 2 minutes, and slowly cooled to 30°C. The annealed template-primer was mixed with 0.1 M DTT, 1.5 M dCTP, 1.5 M dGTP, 1.5 M dTTP, 5 µCi [α-^{35}S]dATP, and 3 units of Sequenase 2.0 (Amersham). The mixture was incubated at 20°C for 2-5 minutes. For each labeling reaction, 2.5 µl of ddATP, ddCTP, ddGTP, or ddTTP was added to four wells in a microwell culture dish, and incubated at 42°C. The labeling mixture was added to each well and incubated at 42°C for 2-5 minutes. Then 4 µl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF) was added to each well. After denaturation with steam for 2 minutes, the mixture in each well was separated on 8 M urea-6% polyacrylamide gel, and detected by autoradiography. The probes used for sequencing are listed as followings: P3, 4, 5, 6, 7, 8, 9, 10, 12, 21, 23, 24, 25, 26, 27, 28, 29 (see Table 4).

Construction of *GAL-NOP2* Strain

Gene disruption was performed according to the method described by Rothstein (Rothstein, 1991). A diploid strain YPH501 was transformed with a *NOP2::HIS3* disruption fragment (Figure 3). Correct integration of this fragment into one *NOP2* locus in the His^+ transformants (YSB12) was confirmed by Southern blotting (not shown).

Figure 3. An intact copy of *NOP2* gene is present in the cells from all of the viable spores. The restriction maps of *NOP2*, *nop2::HIS3* fragment and the probe are as shown. The genomic DNA from the cells germinated from the two spores of one ascus (lane 1 and 2) and the four spores of another ascus (lane 3, 4, 5, and 6) was digested with *Hind*III and analyzed with Southern blotting.

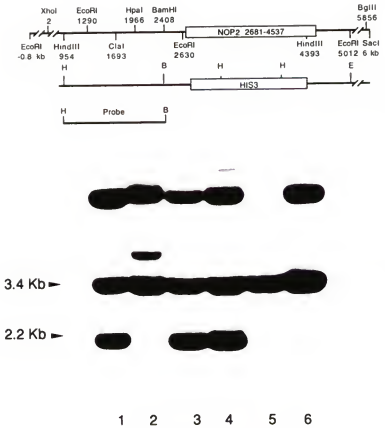

XhoI
2
EcoRI
1290
HpaI
1966
BamHI
2408
BglII
5856
NOP2 2681-4537
EcoRI
-0.8 kb
HindIII
954
ClaI
1693
EcoRI
2630
HindIII
4393
EcoRI SacI
5012 6 kb
H
B
H
H
E
HIS3
H
Probe
B
3.4 Kb
2.2 Kb
1 2 3 4 5 6

Figure 4. Nop2p is expressed in the cells from all of the viable spores. YBH9 and YSB12 are the diploid strains with one *NOP2* locus disrupted and replaced by *LEU2* or *HIS3*, respectively. Total cellular proteins were extracted from the cells germinated from the four spores (a, b, c, and d) of the same ascus, separated on a SDS-polyacrylamide gel, and analyzed by Western blotting. The position of Nop2p is indicated. L: the wild-type haploid L4717. The band with a low molecular weight may be non-specific.

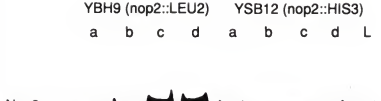
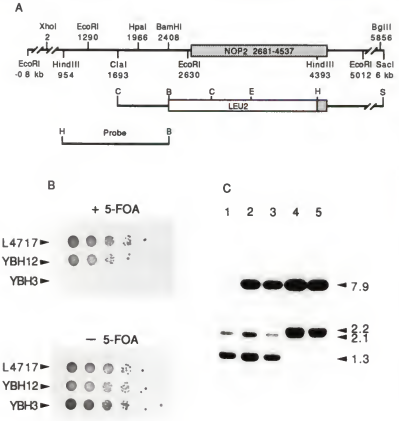

YBH9 (nop2::LEU2)
YSB12 (nop2::HIS3)
a b c d a b c d L
Nop2p

Figure 5. Disruption of NOP2 gene in a haploid L4717 strain. (**A**) The restriction maps of *NOP2*, *nop2::LEU2* fragment and the probe. (**B**) The *nop2::LEU2* strain, YBH3, was dependent upon the plasmid-carried *NOP2* gene for viability. Serial dilutions of L4717, YBH12 and YBH3 were grown on SD medium with or without 5-FOA. YBH12 was constructed by introducing pJPA40 (*NOP2*, *URA3*) into L4717. YBH3 was derived from the disruption of the *NOP2* locus in YBH12 by *nop2::LEU2* fragment. (**C**) Disruption of the *NOP2* locus was confirmed by Southern analysis. Genomic DNA from L4717 (lane 1), and two *NOP2* isolates (lane 2 and 3) and *nop::LEU2* isolates (lane 4: YBH3, and lane 5: YBH4) carrying pJPA40 plasmid were digested with *Eco*RI and analyzed with Southern blotting. The 7.9-kb band corresponds to the linearized pJPA40 plasmid.


A
XhoI
2
EcoRI
1290
HpaI
1966
BamHI
2408
BglII
5856
NOP2 2681-4537
EcoRI
-0 8 kb
HindIII
954
ClaI
1693
EcoRI
2630
HindIII
4393
EcoRI
5012
SacI
6 kb
C
B
C
E
H
S
LEU2
H
Probe
B
B
+ 5-FOA
L4717
YBH12
YBH3
− 5-FOA
L4717
YBH12
YBH3
C
1 2 3 4 5
7.9
2.2
2.1
1.3

However, tetrad dissection revealed that all of the four spores from the same tetrad were viable with two His$^-$ and two His$^+$ spores (not shown). To see if Nop2p was required for the viability of the cells from the spores, Western analysis with an affinity-purified polyclonal antibody APpAb3 to Nop2p was done (Figure 4, YSB12). Nop2p was detected in the cells germinated not only from His$^-$ spores (lanes c and d) but also from His$^+$ spores (lanes a and b) of the same tetrad (Figure 4). Genomic DNA was digested with *Hind*III and analyzed with Southern blotting (Figure 3). The His$^-$ cells from the two spores exhibited a 3.4-kb band, but no 2.2-kb band (Figure 3, lanes 5 and 6), indicating the cells contained only one intact copy of *NOP2*. However, The His$^+$ cells from another two spores showed not only 2.2-kb band but also 3.4-kb band (Figure 3, lane 3 and 4), suggesting that the cells contained copies of both the disrupted and the intact *NOP2*. Considering that this might be a strain-specific phenomenon, several other diploid strains (STX466, 9933D, YSB11 and YJA501) were tested, but with similar results (not shown). Disruption with *NOP2::LEU2* fragment also showed the same result (Figure 4, YBH9).

To avoid this sporulation-related problem, a disruption of the gene was made in a haploid strain in the presence of a plasmid-carried *NOP2* gene. Part of the 5' non-coding region and 92% of *NOP2* open reading frame (ORF) carried on pJPA40 plasmid was replaced by a *Bam*HI/*Hind*III fragment containing the *LEU2* gene, which was isolated from plasmid pJJ282. The *NOP2::LEU2* disruption fragment (Figure 5A) was then linearized with *Xho*I/*Sac*I digestion. The wild type haploid strain L4717 cells were first transformed with *NOP2* gene carried on the pJP40 plasmid (*URA3* marker). The Ura$^+$ transformant (YBH12) was viable in the presence of 5-FOA (Figure 5B), suggesting that the cells could survive by losing the *URA3* plasmid. The YBH12 cells were then transformed with *NOP2::LEU2* fragment (Figure 5A), which, if correctly integrated, should replace 92% of *NOP2* ORF and part of the 5'-non-coding region with *LEU2* gene. One of Leu$^+$/Ura$^+$ transformants (YBH3) was dead in the presence of 5-FOA, suggesting that *NOP2* gene had been disrupted (Figure 5B). To further confirm the correct integration

of *NOP2::LEU2* fragment at the *NOP2* locus, genomic DNA from YBH3 and several other Leu$^+$/Ura$^+$ isolates was digested with *Eco*RI, and analyzed by Southern blotting (Figure 5C). In YBH3 (lane 4) and another isolate (lane 5), a 1.3-kb band was lost, but an extra 2.2-kb band was observed, indicating *NOP2* gene was disrupted and replaced with *LEU2* gene.

To generate a conditionally lethal *nop2* allele, the *NOP2* ORF was placed under the control of a repressible *GAL10* promoter. First, a *Bam*HI/*Xho*I fragment containing the *NOP2* ORF was isolated from pJPA20 plasmid, and subcloned into plasmid pRD53. The *Sac*I/*Xho*I fragment containing *GAL-NOP2* was excised from this subcloned plasmid (pBH46), and cloned into pRS314 plasmid.

The *GAL-NOP2* strain (YBH5) was constructed by using plasmid shuffling technique (Sikorski and Boeke, 1991). The pBH47 plasmid was transformed into YBH3 strain. To replace the original plasmid (pJP40) with the second one (pBH47), the Trp$^+$/Ura$^+$ transformants were replica plated onto SGal plate containing 5-FOA. One of the colonies formed on the plate, YBH5, was used for the depletion analysis.

Cell Growth Curve and Viability Test

YBH5 cells were first grown at 30°C in YPGal liquid medium until mid-log phase. The cells were then collected, washed and resuspended in water. Equal volume of the cells was transferred into YPD and fresh YPGal media, respectively. At different time points, cell densities were measured by using a spectrophotometer (Beckman) at A_{600}. To keep the cells growing in mid-log phase, the cells were diluted into the fresh media whenever cell density reached 0.6-0.8 OD_{600} unit.

The six *nop2* temperature-sensitive strains were grown at 25°C in YPD, and then shifted to 37°C. Cell densities were measured as mentioned above. To test the cell viability after Nop2p depletion, the cells grown in YPD or YPGal medium were collected at the

different time points, diluted to 0.001 OD_{600} unit and then spread 20 µl (about 160 cells) onto YPGal plates. After incubated at 30°C for 2 days, the number of the colonies formed on the plate was counted (Table 5).

Isolation of *nop2* Conditionally Lethal Alleles

***In vitro* mutagenesis of the *NOP2* gene.** A low fidelity polymerase chain reaction (PCR) method (Muhlrad et al., 1992) was used to generate random mutations in *NOP2* gene. A *Kpn*I fragment containing *NOP2* gene was cut out from pJPA29 plasmid and used as the template. Two oligonucleotides were used as the primers: P19A, complementary to the 5' end; and P16, complementary to the 3' end of the gene. Each of four PCR reactions was set up as followings: 20 ng DNA template, 1X reaction buffer (50 mM Tris-HCl, pH 9.0, 50 mM NaCl, and 10 mM $MgCl_2$), 0.4 µM of each primer, 0.1 mM each of three dNTPs with another dNTP at 0.025 mM, 1.25 units of *Taq* DNA polymerase (Promega), with or without 0.15 mM $MnCl_2$. Each reaction with a total volume of 25 µl was overlaid with 100 µl of mineral oil, and run in a Thermocycler (Coy) for 30 cycles (for each cycle: denaturation at 94°C for 90 seconds; annealing at 55°C for 120 seconds; and extension at 72°C for 180 seconds). The PCR products from the four reactions were then pooled and gel-purified. In this study, it was found that the majority of *nop2* alleles generated in the presence of 0.15 mM $MnCl_2$ were non-functional even at the permissive conditions, suggesting that Mn^{2+} caused a much higher error frequency in the PCR reaction.

Preparation of the gapped plasmid. pBH49 plasmid was cut with *Aat*II/*Sna*BI, and the gapped plasmid was gel-isolated. The sequence homology between the gapped plasmid and the PCR products was ~380 bp at the 5' end and ~715 bp at the 3' end. It was found that with only 65 bp sequence homology, an efficient recombination still occurred (Muhlrad et al., 1992).

Construction of the stains containing only mutated *nop2*. Approximately 140 ng of the PCR products and ~60 ng of the gapped plasmid were cotransformed into YBH3 by the lithium acetate method. Muhlrad et al. found that homologous recombination between a gapped plasmid and a linear fragment following cotransformation into yeast is efficient (Muhlrad et al., 1992), so that *in vitro* ligation is not necessary. Plasmid shuffling was performed as described above. As controls, the gapped plasmid or the PCR products alone were also transformed into YBH3. Only 5-10 colonies/plate formed with the gapped plasmid alone, while no colony formed with the PCR products alone.

Isolation of conditionally lethal strains. Colonies formed in the presence of 5-FOA were replica plated onto SD medium, and incubated at 14°C, 25°C and 37°C. Two other restrictive conditions, 6% ethanol or 3% formamide were also tested at 25°C (Aguilera and Benitez, 1986; Aguilera, 1994).

Recovery of the plasmids. The plasmid DNA was recovered from the yeast cells, amplified in *E. coli* DH5α, and verified by restriction digestion. The ORF of *nop2* alleles was cut out by digestion with *Eco*RI, gel-isolated, and subcloned into pBH49 plasmid at the same sites.

Mapping of the Mutations in the Six *nop2* Alleles

The six *nop2* alleles carried on pRS313 were manually sequenced and the predicted amino acid substitutions were located (see Figure 19). To identify the mutations contributing to temperature-sensitive (ts) phenotype, the multiple point mutations in each allele carried on pRS313 were separated into several groups by restriction digestion with *Bsg*I, *Mfe*I or *Spe*I. The restriction fragments containing the mutations were gel-isolated and exchanged with the fragments from *NOP2*. After ligation, the subclones were amplified in *E. coli* DH5α. The subcloned plasmids containing one or several groups of

the point mutations were transformed into YBH3 strain. After plasmid shuffling, the phenotypic effect of the mutations contained in each subcloned plasmid was analyzed by incubating the cells under the restrictive condition (37°C). The subcloned plasmids were constructed as described in Table 3.

SDS-PAGE and Immunoblotting

Total cellular proteins were extracted in the presence of trichloroacetic acid, precipitated, and separated on 10.5% polyacrylamide gel by SDS gel electrophoresis (SDS-PAGE), and analyzed by immunoblotting with affinity-purified polyclonal anti-Nop2p antibody (APpAb3) (de Beus et al., 1994). A monoclonal antibody (1G1) against the poly (A) binding protein (Pab1P) (Anderson et al., 1993) was used to probe the same blot.

Measurement of Ribosomes and Polysomes

The levels of ribosomal subunits, monoribosomes and polyribosomes were measured as described previously (Baim et al., 1985). Cells in mid-log phase were collected, washed with lysis buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 30 mM $MgCl_2$) containing 100 µg/ml cycloheximide, and broken by vortexing with acid-washed glass beads. The lysate was centrifuged at 10,000*g* at 4°C for 5 min., and the supernatant was loaded onto 15%-50% sucrose gradient. The gradient was centrifuged at 40,000 rpm (285,000*g*) for 2.5 h at 4°C in SW41Ti rotor (Beckman Instruments, Inc.). After centrifugation, the gradient was analyzed by using an absorbance detector (ISCO UA-5). The polysome curves were traced by using Freehand software.

Analysis of Pre-rRNA Processing by Pulse-Chase Labeling

Approximately 25 OD_{600} units of cells in the mid-log phase (OD_{600}~0.5) were collected, and resuspended in 5 ml of the synthetic minimal media. The cells were pulse labeled with 250 µCi [*methyl*-^{3}H]methionine or [^{3}H]uracil at 25°C for 3 min. A large excess of unlabeled methionine (5 mM) or uracil (2 mM) was added. At the different chase time points, 1 ml of cells was collected and centrifuged for 10 seconds. Cell pellets were immediately frozen in dry ice and stored at -70°C for further analysis.

Total RNAs were extracted as described (Kohrer and Domdey, 1991). Briefly, cells were treated with 1% SDS and phenol at 65°C for 5 min. After centrifugation, the supernatant was extracted with phenol::$CHCl_3$ and $CHCl_3$ sequentially. The RNAs were precipitated by using 0.3 M NaOAc plus 2.5 volume of 100% ethanol.

To increase the relative ratio of signal to noise, [^{3}H]uracil labeled RNAs were passed through oligo (dT)-cellulose (Boehringer Mannheim). The supernatant was transferred to a fresh tube and the RNA was ethanol precipitated, washed and dried. The RNAs were separated on 1.2% agarose-glyoxal gel or 12% polyacrylamide-urea gel (Ausubel et al., 1993). The gel was treated with En^{3}Hance (DuPont NEN), dried and exposed to X-ray film.

Northern Analysis of rRNAs

Total RNAs were extracted, separated on 1.2% agarose-glyoxal gel, and transferred to Hybon nylon membrane (Amersham). The blot was probed with the [^{32}P] labeled oligonucleotides specific for the sequences in the internal transcribed spacer (ITS) 1 and 2, and in 18S and 25S rRNAs, and exposed to X-ray film. Before reprobing, the blot was submerged into boiling 0.5% SDS, cooled to room temperature, and washed with water. The oligonucleotides used as probes are listed in Table 4.

Primer Extension

Standard primer extension was performed as described below. Approximately 1 μg of RNA template and 1 pmol of primer in the buffer (25 mM Tris-HCl, pH 8.3, 25 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 0.25 mM spermidine) were denatured at 70°C for 10 min. and annealed at 42°C for 20 minutes. Labeling was done for 1 minute at 42°C in the presence of the followings: 0.5 mM each of dCTP, dGTP, and dTTP, 10 μCi of [α-^{35}S]dATP, 5 units of avian myeloblastosis virus reverse transcriptase (Promega) and 20 units of RNase Block II (Stratagene). Extension was done for 10 minutes at 42°C after adding dATP to 1 mM. The reaction was stopped by placing the reaction mixture on ice and adding 10 mM EDTA, and the nucleic acids were precipitated with ethanol prior to electrophoresis in 8 M urea-6% polyacrylamide gel.

For concentration dependent pause (CDP) assay (Maden et al., 1995), the same procedure as the standard primer extension was performed, but with the concentration of dNTPs at 12.5 μM each, and 20 μCi of [α-^{35}S]dATP during labeling. Extension was done with all four dNTPs at 4, 20, and 100 μM. For partial alkaline hydrolysis of RNA (Sirum-Connolly and Mason, 1993), 2 μg RNAs were digested in 20 μl of 50 mM Na_2CO_3, pH 9.0 at 90°C for 6 minutes before standard primer extension.

In vitro Methylation Analysis

A 3.8-kb *ApaI*/*Bam*HI fragment containing T7 or T3 promoter followed by 27S rDNA sequence was linearized from pBluescript KS$^+$ or SK$^+$. *In vitro* transcription was performed by using a commercially available kit (Promega). The reaction condition was set up according to the manufacturer's protocol. Both T3 and T7 RNA polymerase were successfully used. The reaction product was analyzed on a 0.8% agarose-glyoxal gel.

Nop2p was obtained by immunoprecipitation (IP) with a monoclonal anti-Nop2p antibody (D68) (Wu, P., B. Hong, J. S. Brockenbrough, and J. P. Aris, unpublished results). When the IP pellet was analyzed by SDS-PAGE and stained with coomassie blue, a band corresponding to the position of Nop2p was seen (not shown).

In vitro methylation reactions were performed as described (Segal and Eichler, 1991). The following components were contained in the reaction: 50 mM Tris-HCl (pH 8.0), 0.4 mM EDTA, 5 mM DTT, 0.4 mg/ml bovine serum albumin (BSA), 50 mM KCl, 2.0 μM [^{3}H]-S-adenosyl-methionine (NEN), ~50 μM 27S rRNA, 40 units of RNase Block (Stratagene), and IP-purified Nop2p. The mixture with the total volume of 100 μl was incubated at 30°C for one to two hours. Considering the possible effects of secondary structure, denaturation of 27S rRNA by boiling before adding to the reaction was also tested. The reaction product was treated with phenol/chloroform. In the presence of 20 μg of glycogen, the RNA was precipitated with 0.5 volume of 7.5 M ammonium acetate and 2.5 volume of 100% ethanol. The RNA was separated on a 0.8% agarose-glyoxal gel. The incorporated isotope was detected with fluorography.

RNase Protection Assay

RNase protection was done using a modification of a previously described method (Segal and Eichler, 1991). RNA was extracted from L4717 or YBH5 grown in SD for 8.5 hours, pulse labeled with [*methyl*-^{3}H]methionine for 3 minutes, and chased for 4 minutes, as described above. [^{3}H]labeled RNA from ~7 OD_{600} units of culture (~30 pmol RNA) plus 300 pmol of oligonucleotide probe were denatured in 40% formamide, 40 mM PIPES-HCl, pH 6.4, 400 mM NaCl, 1 mM EDTA for 15 minutes at 85°C. The oligonucleotides used for protection are as follows: oligo 6, oligo 7, and oligo 8 (Table 4). After cooling to 25°C, single stranded RNA was digested with RNase A (20 μg/ml) and RNase T1 (3 μg/ml) freshly added to 300 mM NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA.

Digestions were inactivated with SDS and proteinase K, supplemented with 10 µg yeast tRNA, extracted with phenol-chloroform, ethanol precipitated, washed with 70% ethanol, and dried. Samples were electrophoresed on 12% polyacrylamide/7M urea gels. Gels were fixed and treated with En^{3}Hance (DuPont). Following fluorography, sections of dried gels were excised, digested with 1 ml of Scintigest (Fisher Scientific) at 37°C overnight in tightly capped glass vials. Samples were neutralized by the addition of 100 µl of glacial acetic acid, mixed with Scintiverse E (Fisher Scientific), and subjected to scintillation counting for a sufficient length of time (0.5 - 8 hours per determination) to yield an error of ± 2% of the mean DPM value at a confidence level of 95%.

Immunoprecipitation Analysis

The buffers used in immunoprecipitation are listed as followings: IP buffer: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 2 mM NaN_3. Lysis buffer-C: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 2 mM NaN_3, 5 mM $MgCl_2$, 0.1% NP-40 (Calbiochem), protease inhibitor cocktails in water (PIC-W) (1 mM benzamide, 0.5 µg/ml leupeptin, 1.5 µM bestatin in ddH_2O), protease inhibitor cocktails in dimethylsulfoxide (PIC-D) (0.5 mM phenylmethylsulfonyl Fluoride, 1.5 µM pepstatin A, 1 µg/ml chymostatin in dimethylsulfoxide), 1 mM DTT, 5 mM vanadyl ribonucleoside complex. Pre-treatment buffer: 100 mM Tris-HCl (pH 9.0), 50 mM DTT, 5 mM EDTA. Lysis buffer-N: 20% Ficoll, 20 mM KP_i, pH 6.5, 1 mM $MgCl_2$, PIC-D and PIC-W. Cushion buffer: 30% Ficoll 400, 20 mM KP_i, pH 6.5, 1 mM $MgCl_2$, PIC-W, and PIC-D. Washing buffer: 50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 1 mM EDTA, and 2 mM NaN_3, 5 mM $MgCl_2$, 0.1% NP-40, PIC-W, and PIC-D.

Monoclonal anti-Nop1p (A66) and anti-Nop2p (D68), and polyclonal anti-Nop2p (APpAb3) antibodies were used. Binding of antibodies to protein-G sepharose (Pharmacia) was performed as followings: about 20 µl protein-G sepharose beads were

washed with PBS buffer (2 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.5 mM KCl, 140 mM NaCl, pH 7.2) plus 0.1% NP-40; after adding 5 µl of either A66 or D68, or 8 µl of APpAb3, the mixture was incubated at the room temperature (~25°C) for 60 minutes; the beads were then washed three times with PBS buffer plus 0.1% NP-40.

For each IP reaction, 5 OD_{600} units of L4717 cells in the mid-log phase were used. An overnight culture of L4717 cells was diluted into fresh YPD to an OD_{600}~0.1. After two doublings (OD_{600}~0.4) at 30°C, the cells were collected and washed with ddH_2O. For protein labeling, the cells were incubated in 10 ml of SD medium (without methionine) at 30°C for 1 hour. The cells were then collected and labeled in 1 ml of SD medium with ~25 µCi of [^{35}S]methionine at RT for 30 minutes. For RNA labeling, the cells were incubated in 10 ml of low-phosphorus-YPD (LP-YPD) at 30°C for 1 hour. Then the cells were labeled in 1 ml of LP-YPD with ~30 µCi of ^{32}P-phosphate at RT for 30 minutes.

For the whole cell lysate, labeled cells in 100 µl of lysis buffer-C were broken by vortexing with RNase-free glass beads for 5 minutes at 4°C. The supernatant was used for IPs.

For nuclear preparation, labeled cells were treated with 100 µl of pre-treatment buffer. After washing with ddH_2O and 1 M sorbitol, the cell wall was subjected to enzymatic digestion. For one OD_{600} of cells, 20 µg of Zymolyase 100T (ICN) and 1µl of glusulase (DuPont) were used. The digestion reaction was set up in 50 µl of 1 M sorbitol at RT for 30 minutes. The reaction was stopped by spinning for 30 seconds at RT at 7,000 rpm (4,000*g*), and the pellet was washed with cold 1 M sorbitol. Spheroplasts were homogenized in the lysis buffer-N using a Dounce homogenizer with 10 strokes at RT. The homogenate was centrifuged at 5,000 rpm (2,300*g*) in an SW50.1 rotor (Beckman) at 2°C for 5 minutes. The supernatant was then loaded onto 1 ml of the cushion buffer. The nuclei in the supernatant were obtained by being centrifuged at 22,000 rpm (45,300*g*) in an SW50.1 rotor at 2°C for 60 minutes. The nuclear pellet was resuspended in 0.5 ml of lysis

buffer-C and sonicated. After spinning at top speed in a microfuge at 4°C for 5 minutes, the supernatant was used for IP.

The supernatant from the whole cell lysate or from the nuclear preparation was mixed with the protein-G sepharose-antibody beads at 4°C for 1 hour with rotation. The beads were washed five times with washing buffer.

For protein analysis, the beads were resuspended in SDS-PAGE sample buffer (4% SDS, 20% glycerol, 0.1 M DTT, 83 mM Tris, 127 mM Tris-HCl, 0.001% bromophenol blue, pH 8.0), and boiled for 2 minutes. After a brief spin, the supernatant was loaded onto 10% SDS-PAGE, and analyzed by electrophoresis.

For analysis of RNA-protein interactions, the RNAs were extracted from the beads as follows. Beads were resuspended in 150 µl of IP buffer plus 1% SDS. In the presence of tRNA carrier, RNAs were extracted by 150 µl of phenol::$CHCl_3$ at 37°C for 15 minutes with vortexing for 1 minute every 5 minutes, and precipitated by ethanol. The extracted RNAs were separated on 7 M urea-6% polyacrylamide gel electrophoresis. The signals were detected by autoradiography.

Screening for High Copy Suppressors

A yeast library carried on 2 µm plasmid with *URA3* marker was kindly given by Dr. Ruben Herriquez (Sandoz Pharmaceuticals AG). The library with an average of 10-kb inserts was prepared by *Sau3*A partial digestion and size fractionation. The library plasmids were transformed into *E.coli* DH5α. Plasmid maxi-preparation was done as follows. An overnight culture of *E.coli* carrying plasmids was collected and treated with 1.25 mg/ml of Lysozyme (Sigma) in 40 mls of GTE buffer (25 mM Tris, 10 mM EDTA, 50 mM glucose, pH 8.0) at RT for 10 minutes. Cells were disrupted with 80 mls of 0.2 N NaOH plus 1% SDS on ice for 15 minutes. After adding 60 mls of 3 M KOAc (pH 5.0), the mixture was placed on ice for 15 minutes, and centrifuged at 4°C for 10 minutes at

5,500 rpm (4,000*g*) in Sorvall GSA rotor. DNA in the supernatant was precipitated by adding 0.6 volumes of isopropanol, and centrifuged at 4°C for 10 minutes at 4,000*g*. Pellet was dissolved in TE buffer, and mixed with 50 mls of 44% of CsCl plus 33% of ethidium bromide in quick-seal ployallomer tubes (Beckman). The mixture was centrifuged at 20°C for 24 hours at 50,000 rpm (242,000*g*) in a VTi50 rotor. The bottom band containing plasmid DNA in CsCl gradients was collected and treated with ddH_2O-saturated 1-butanol. DNA was obtained by ethanol precipitation. The library was then transformed into each of the six ts strains. In this study, it was found that almost no colony was formed if the transformants were incubated at 37°C immediately after transformation. Therefore, the transformants were incubated at 25°C for 24 hours before shifting to 37°C. Since yeast genome size is about 14 Mb, it can be estimated that with this library, a minimum of 1,400 colonies for each ts strain is required for covering the whole yeast genome. The library plasmids were transformed into each of the six ts strains. The plasmids were recovered from the colonies formed at 37°C, and amplified in *E. coli* DH5α. The suppression effect of the recovered plasmids was tested. Whether the recovered plasmids contained inserts was determined by restriction digestion with *Pst*I, *Xba*I, *Nsi*I and *Eco*RV, which cut the plasmid backbone. Presence of URA3 marker in the recovered plasmids was determined by digestion with *Eco*RV/*Nsi*I. Among about 100 plasmids recovered, only one contained a 3.7-kb insert. The *Sac*I/*Bgl*II fragment containing 3.0-kb of the insert was then subcloned into pRS426 plasmid at the *Sac*I/*Bam*HI sites, and its phenotypic effect was re-tested. Sequencing of the insert was done with the reverse primer, which was complementary to the polylinker 5' upstream to the insert. Another primer, JA36, complementary to the sequence of 25S rDNA, was also used for sequencing. Sequencing with -20 and -40 primers complementary to the polylinker 3' downstream to the insert was not successful. Whether the suppression was allele-specific was analyzed by testing the phenotypic effect of the insert in each of the six ts strains.

Table 2. Yeast Strains

Strain	Genotypic description	Source
L4717	*MATa*, *ade2*, *can1-100*, *his3-11,15* , *leu2-3,-112*, *trp1-1*, *ura3-1*, *GAL*$^+$	C. A. Style G. R. Fink
L4718	*MAT*α, ade2, *can1-100*, *his3-11,-15*, *leu2-3*,-112, *trp1-1*, *ura3-1*, *GAL*$^+$	C. A. Style G. R. Fink
YBH3	L4717, *nop2::LEU2*, pJPA40	This study
YBH9	YPH501, *nop2::LEU2*	This study
YBH12	L4717, pJPA40	This study
YBH15	L4717, *nop2::LEU2*, pBH60	This study
YBH16	L4717, *nop2::LEU2*, pBH67	This study
YBH17	L4717, *nop2::LEU2*, pBH72	This study
YBH18	YBH15, pJPA29	This study
YBH20	L4717, *nop2::LEU2*, pBH55	This study
YBH21	L4717, *nop2::LEU2*, pBH74	This study
YBH23	L4717, *nop2::LEU2*, pBH77	This study
YBH26	YBH20, pJPA29	This study
YBH27	YBH16, pJPA29	This study
YBH28	YBH17, pJPA29	This study
YBH29	YBH21, pJPA29	This study
YBH30	YBH23, pJPA29	This study
YBH31	L4717, *nop2::LEU2*, pBH49	This study
YBH32	L4717, *nop2::LEU2*, pBH56	This study
YBH33	L4717, *nop2::LEU2*, pBH57	This study
YBH34	L4717, *nop2::LEU2*, pBH58	This study
YBH35	L4717, *nop2::LEU2*, pBH59	This study
YBH36	L4717, *nop2::LEU2*, pBH61	This study

Table 2. Continued

Strain	Genotypic description	Source
YBH37	L4717, *nop2::LEU2*, pBH62	This study
YBH38	L4717, *nop2::LEU2*, pBH63	This study
YBH39	L4717, *nop2::LEU2*, pBH64	This study
YBH40	L4717, *nop2::LEU2*, pBH65	This study
YBH41	L4717, *nop2::LEU2*, pBH66	This study
YBH42	L4717, *nop2::LEU2*, pBH68	This study
YBH43	L4717, *nop2::LEU2*, pBH69	This study
YBH44	L4717, *nop2::LEU2*, pBH70	This study
YBH45	L4717, *nop2::LEU2*, pBH71	This study
YBH46	L4717, *nop2::LEU2*, pBH73	This study
YBH47	L4717, *nop2::LEU2*, pBH75	This study
YBH48	L4717, *nop2::LEU2*, pBH76	This study
YBH49	L4717, *nop2::LEU2*, pBH78	This study
YJA501	*MAT*a/α, *leu2Δ2, ura3-52, GAL2*	J. Anderson
YPH501	*MAT*a/α, *ade2-101a, his3Δ200, leu2Δ1, lys2-801a, trp1Δ63, ura3-53, GAL*$^+$	R. S. Sikorski
YSB-11	Diploid from cross of L4717 and L4718	J. S. Brockenbrough
YSB12	YSB-11, *nop2::HIS3*	J. S. Brockenbrough
STX466	*MAT*a/α, *ade2-1o*, *his3Δ200*, *lys2-801a*, *trp1Δ*, *ura3-52, gal*	Yeast genetic stock center
9933-13A	*MAT*a, *his3Δ200*, *leu2-3,-112, trp1Δ1*, *ura3-52*, *GAL*	G. R. Fink
9933-20C	*MAT*α, *his3Δ200*, *leu2-3,-112, trp1Δ1*, *ura3-52*, *GAL*	G. R. Fink
9933D	Diploid from cross of 9933-20C and 9933-13A	This study

Table 3. Plasmids

Plasmid	Relevant functional DNA	Comments
pAH6	*NOP2 HIS3 CEN6*	*Sac*I fragment containing *NOP2* cloned at the same sites of pRS313
pBH46	*GAL10-NOP2 URA3 CEN6*	*Bam*HI/*Xho*I fragment containing *NOP2* from pJPA20 cloned in the same sites of pRD53
pBH47	*GAL10-NOP2 TRP1 CEN6*	*Sac*I/*Xho*I fragment containing *GAL10-NOP2* from pBH46 cloned in the same sites in pRS314
pBH49	*NOP2 HIS3 CEN6*	Derived from pAH6 with deletion of *Cla*I fragment from the upstream of *NOP2*
pBH55	*nop2-3 HIS3 CEN6*	*Eco*RI fragment containing *nop2-3* cloned at the same sites of pBH49
pBH56	*nop2-3a HIS3 CEN6*	*Mfe*I fragment from *nop2-3* cloned at the same sites of pBH49
pBH57	*nop2-3b HIS3 CEN6*	*Mfe*I fragment from *NOP2* cloned at the same sites of pBH55
pBH58	*nop2-3c HIS3 CEN6*	*Spe*I fragment from pBH55 cloned at the same sites of pBH49
pBH59	*nop2-3d HIS3 CEN6*	*Spe*I fragment from pBH49 cloned at the same sites of pBH55
pBH60	*nop2-4 HIS3 CEN6*	*E.co*RI fragment containing *nop2-4* cloned at the same sites of pBH49
pBH61	*nop2-4a HIS3 CEN6*	*Bsg*I/*Cla*I fragment from *nop2-4* cloned at the same sites of pBH49
pBH62	*nop2-4b HIS3 CEN6*	*Bsg*I/*Cla*I fragment from *NOP2* cloned at the same sites of pBH60
pBH63	*nop2-4c HIS3 CEN6*	*Mfe*I fragment from *NOP2* cloned at the same sites of pBH62
pBH64	*nop2-4d HIS3 CEN6*	*Mfe*I fragment from *nop2-4* cloned at the same sites of pBH49

Table 3. Continued

Plasmid	Relevant functional DNA	Comments
pBH65	*nop2-4g HIS3 CEN6*	Derived from pBH62. *Mfe*I fragment deleted, and the gapped plasmid ligated at the same site. *Cla*I/*Mfe*I fragment from pBH49 cloned at the same sites. *Mfe*I fragment from *nop2-4* then cloned at the same site.
pBH66	*nop2-4h HIS3 CEN6*	Derived from pBH49. *Mfe*I fragment deleted, and the gapped plasmid ligated. *Cla*I/*Mfe*I fragment from pBH63 cloned at the same sites. *Mfe*I fragment from *nop2-4* then cloned at the same site.
pBH67	*nop2-5 HIS3 CEN6*	*E.co*RI fragment containing *nop2-5* cloned at the same sites of pBH49
pBH68	*nop2-5a HIS3 CEN6*	*Mfe*I fragment from *nop2-5* cloned at the same sites of pBH49
pBH69	*nop2-5b HIS3 CEN6*	*Mfe*I fragment from *NOP2* cloned at the same sites of pBH67
pBH70	*nop2-5c HIS3 CEN6*	*Spe*I fragment from pBH67 cloned at the same sites of pBH49
pBH71	*nop2-5d HIS3 CEN6*	*Spe*I fragment from pBH49 cloned at the same sites of pBH67
pBH72	*nop2-6 HIS3 CEN6*	*E.co*RI fragment containing *nop2-6* cloned at the same sites of pBH49
pBH73	*nop2-6a HIS3 CEN6*	*Bsg*I/*Mfe*I fragment from *nop2-6* (one of two *Mfe*I sites lost from the mutation) cloned at the same sites of pBH49
pBH74	*nop2-9 HIS3 CEN6*	*E.co*RI fragment containing *nop2-9* cloned at the same sites of pBH49
pBH75	*nop2-9a HIS3 CEN6*	*Mfe*I fragment from *nop2-9* cloned at the same sites of pBH49
pBH76	*nop2-9b HIS3 CEN6*	*Mfe*I fragment from *NOP2* cloned at the same sites of pBH74

Table 3. Continued

Plasmid	Relevant functional DNA	Comments
pBH77	*nop2-10 HIS3 CEN6*	*Eco*RI fragment containing *nop2-10* cloned at the same sites of pBH49
pBH78	*nop2-10a HIS3 CEN6*	*Bsg*I/*Mfe*I fragment from *nop2-10* (one of two *Mfe*I sites lost from the mutation) cloned at the same sites of pBH49
pJJ282	*LEU2*	Jones, J. S. and L. Prakash
pJPA20	*NOP2*	*Eco*RI fragment containing *NOP2* cloned into pBluescript SK^+
pJPA29	*NOP2 TRP1 CEN6*	*Xho*I/*Bgl*II containing *NOP2* cloned at *Xho*I/*Bam*HI of pRS314
pJPA40	*NOP2 URA3 CEN6*	*Hpa*I/*Kpn*I fragment containing *NOP2* plus 715 bp of 5' flanking sequence and 456 bp of 3' flanking sequence from pJPA20 cloned into pRS316
pRD53	*GAL1, 10*	R. J. Deshaies
pRS426	2μm *URA3*	T. Christianson

Table 4. Oligonucleotides

Name	Sequence
JA36	5'-GGTGGGTTAGTCGATCC-3'
Oligo1 (18S)	5'-AGCCATTCGCAGTTTCACTG-3'
Oligo2 (ITS1-U)	5'-GCACAGAAATCTCTCACCGT-3'
Oligo3 (ITS1-D)	5'-TCCAGTTACGAAAATTCTTG-3'
Oligo4 (ITS2-D)	5'-GCCTAGACGCTCTCTTCTTA-3'
Oligo5 (25S)	5'-TACTAAGGCAATCCGGTTGG-3'
Oligo6	5'-CGCATAGACGTTAGACTCCTTGGTCCGTGTTTCAAGACGG-3'
Oligo7	5'-CCCTATTAGTGGGTGAACAATCCAACGCTTACCG-3'
Oligo8	5'-CGGTCTAAACCCAGCTCATGTTCCCTATTAGTGGG-3'
Oligo9 (A)	5'-CCTTAGAGCCAATCCTTATC-3'
Oligo10 (B)	5'-CTGTTGACGTGGAACCTTTC-3'
Oligo11 (C)	5'-CTGATGAGCGTGTATTCCGG-3'
Oligo12 (D)	5'-CAGGAACCAGCTACTAGATG-3'
Oligo13 (E)	5'-CACTTTCATTACGCGTATGG-3'
Oligo14 (JA22)	5'-CATCAGTAGGGTAAAACTAAC-3'
Oligo15 (JA30)	5'-TGTTCTACTGGAGATTTCTG-3'
Oligo16 (JA31)	5'-TAGGGACAGTGGGAATCTCG-3'
P3	5'-GCGGAGAACATCCAAC-3'
P4	5'-AGATCGGTCCATCTTCG-3'
P5	5'-GGTTGCATGTTCTCCGC-3'
P6	5'-CCTTGAAGACCAGAAGG-3'
P7	5'-CGAAGATGGACCGATCT-3'
P8	5'-GTACCGGAACATGGGGC-3'
P9	5'-TGAAGCAGGATTGGTGG-3'

Table 4. (continued)

Name	Sequence
P10	5'-TCATCCACCAATCCTGC-3'
P12	5'-CTATGCTAACATGATGC-3'
P16	5'-TTCCTTCTACCGGTACC-3'
P19A	5'-GCCGGACGATAACGTGC-3'
P21	5'-CTAAGTCTCTCCTTCTG-3'
P23	5'-AAGAACAAGCAAGCCGC-3'
P24	5'-GTACCGAGAAGGACTTC-3'
P25	5'-GAATTCCCCTTGTGTTG-3'
P26	5'-AGCGAAAACACAACCTG-3'
P27	5'-GAATCAATGGCGGATAG-3'
P28	5'-TGCTCTAGATCCTCATG-3'
P29	5'-ACCGAATGTCAAGTTAG-3'
Reverse	5'-AACAGCTATGACCATG-3'
-20	5'-GTAAAACGACGGCCAGT-3'
-40	5'-GTTTTCCCAGTCACGGAC-3'

Table 5. Cell Viability after Nop2p Depletion

Time of Depletion (Hour)	Colony Number (Depleted)	(Non-depleted)	Survivability (%)
0	157	155	101
1	152	150	101
2	149	154	97
4	151	155	97
8	147	156	94
10	144	159	91
23	145	162	90
25	140	155	90

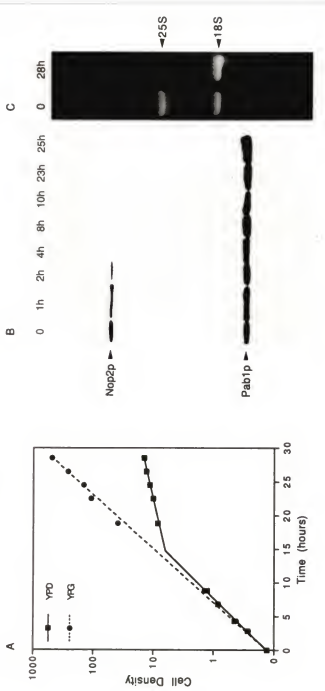
RESULTS

Depletion of Nop2p

Repression of *NOP2* Impaired Cell Growth

When expression of a gene is repressed, the protein encoded by the gene will be depleted because of protein turnover and dilution by cell division. In this way, a deficit in the protein function will be revealed. To repress the activity of the *GAL* promoter and deplete Nop2p, YBH5 (*GAL-NOP2*) cells were shifted from YPGal to YPD medium. During the first 8 hours after the medium-shift, YBH5 cells grew at a similar rate in both media. However, after 8 hours' incubation, the cell growth in YPD medium was progressively impaired, as compared with that in YPGal medium (Figure 6A). The doubling time in YPD medium increased about fivefold from 2.5 hours at the medium-shift to 12.5 hours after the medium-shift for 28 hours. To confirm that Nop2p in the YBH5 cells grown in YPD medium was indeed depleted, Western blotting with an antibody to Nop2p was performed. At the 2 hour time point after medium-shift, the Nop2p level was significantly reduced. After 4 hours' depletion, Nop2p was not detectable (Figure 6B). The cytoplasmic poly(A) binding protein Pab1p level was not affected during the incubation in YPD, suggesting that Nop2p was specifically depleted (Figure 6B). Since a significant depletion of Nop2p occurred before the cell growth slowed down, the cell growth was dependent upon the function of Nop2p.

Figure 6. The effect of Nop2p depletion on cell growth and the level of 25S rRNA. **(A)** Growth curves of YBH5 (*GAL-NOP2*) cells. The cells grown in YPGal were transferred to either fresh YPGal or YPD medium (0 time point). At the indicated time points, cell density was measured at A_{600}. **(B)** Depletion of Nop2p was confirmed by Western analysis. YBH5 cells grown in YPD medium were collected at the indicated time points, and total cellular proteins were extracted and analyzed by Western blotting with an antibody to Nop2p. The same blot was re-probed with the antibody to the poly(A) binding protein Pab1p. **(C)** Depletion of Nop2p caused a reduction in the 25S rRNA levels. Total RNAs were extracted from YBH5 cells grown in YPD medium for 0 and 28 hours, separated on an agarose-glyoxal gel, and stained with ethidium bromide.


A
YPD
YPG
Cell Density
1000
100
10
1
0
0
5
10
15
20
25
30
Time (hours)
B
0
1h
2h
4h
8h
10h
23h
25h
Nop2p
Pab1p
C
0
28h
25S
18S

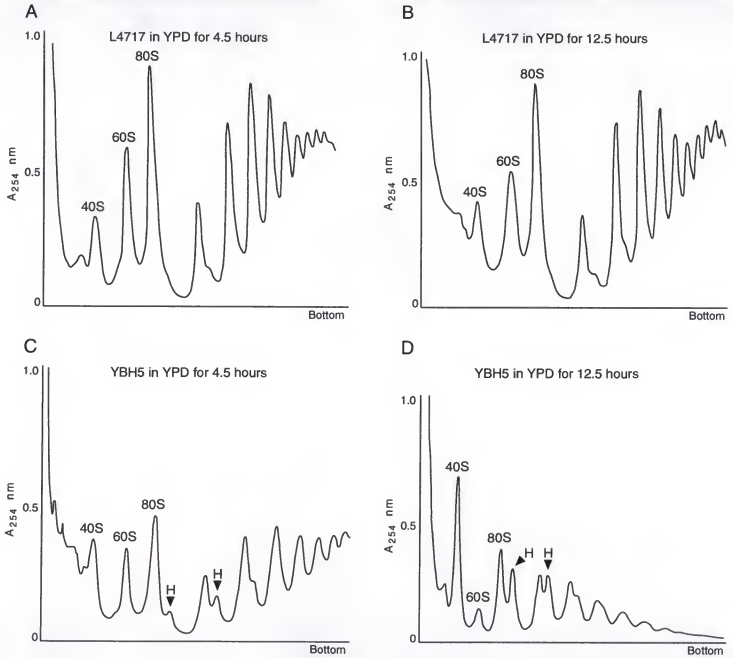
Depletion of Nop2p Caused a Reduction in 25S rRNA and 60S Ribosomal Subunits

The nucleolar localization of Nop2p (de Beus et al., 1994) was a clue that it might be related to nucleolar function. The essentiality of this protein to cell growth and viability might be due to its involvement in the ribosome synthesis, one of the main functions in the nucleolus (Hadjiolov, 1985).

To investigate this possibility, the relative levels of 18S and 25S rRNAs in Nop2p-depleted cells were measured. Prior to Nop2p depletion (at 0 time point), the ratio of 25S and 18S rRNAs was close to 1:1 (Figure 6C). However, after 28 hours' depletion of Nop2p, 25S rRNA level was significantly reduced and the ratio of 25S to 18S rRNAs was much less than 1:1 (Figure 6C). Over 90% of YBH5 cells grown in YPD for 28 hours were still viable (Table 5). Therefore, the observed reduction of 25S rRNA was not caused by cell death. Since 25S rRNA is a structural component of 60S ribosomal subunit, these results suggest that Nop2p is involved in synthesis of the 60S ribosomal subunit.

To confirm the role of Nop2p in ribosome synthesis, the effect of Nop2p depletion on the levels of ribosomal subunits and polyribosome was analyzed. A typical, normal polysome profile was shown in the control L4717 cells (Figure 7A and B). In contrast, depletion of Nop2p for 4.5 hours caused a reduction in the level of 60S subunit relative to that of 40S subunit. The peak of 80S monomer, which consists of the 40S and 60S subunit associated with an mRNA, was accordingly lowered. Three small extra peaks appeared immediately following the peaks of 80S monomer and the first two polysome (Figure 7C). These extra peaks possibly represent halfmer polysomes, which contain the mRNA-associated polysomes plus a preinitiation complex consisting of 40S subunit with the initiation factors (Helser et al., 1981; Moritz et al., 1990). A similar abnormal polysome profile was observed when synthesis of 60S ribosomal subunit was aborted by depletion of large subunit proteins L1 or L16 (Moritz et al., 1990; Deshmukh et al., 1993).

Figure 7. Depletion of Nop2p causes a significant reduction in the level of the 60S ribosomal subunit. YBH5 (**C** and **D**) and the wild-type L4717 (**A** and **B**) were shifted from YPGal to YPD medium, and collected after 4.5 hours (**A** and **C**) or 12.5 hours (**B** and **D**) after the medium shift. The ribosomal subunits, 80S monoribosome, and polyribosomes in the cell lysate were separated on sucrose density gradients and detected by measurement of A_{254} values. Halfmer ribosomes are labeled as H.

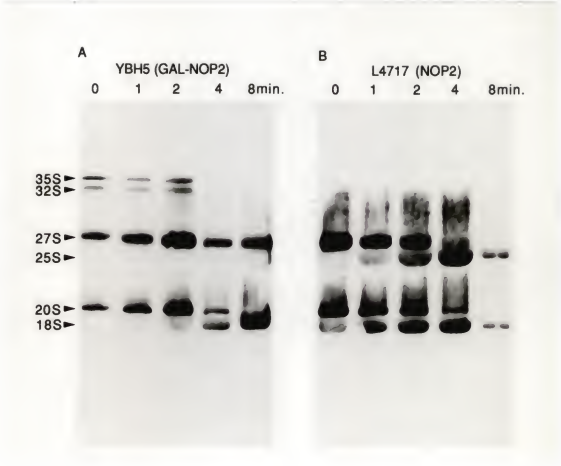

A
L4717 in YPD for 4.5 hours
1.0
0.5
0
A_{254} nm
40S
60S
80S
Bottom
B
L4717 in YPD for 12.5 hours
1.0
0.5
0
A_{254} nm
40S
60S
80S
Bottom
C
YBH5 in YPD for 4.5 hours
1.0
0.5
0
A_{254} nm
40S
60S
80S
H
H
Bottom
D
YBH5 in YPD for 12.5 hours
1.0
0.5
0
A_{254} nm
40S
60S
80S
H
H
Bottom

Since the growth rate of the Nop2p-depleted cells was similar to that of the non-depleted cells during the first 8 hours of growth in YPD, the aberrant polysome profile was not due to a secondary effect of slow growth. After the medium-shift to YPD for 12.5 hours, the level of the 60S subunit was significantly reduced while the 40S subunit accumulated (Figure 7D). The reduction of 80S monomer was more significant and the halfmer peaks were more obvious, suggesting that the shortage of the 60S subunit was much more severe after a longer period of depletion (Figure 7D). These observations indicate that Nop2p is required for synthesis of the 60S ribosomal subunit.

Nop2p is Required for Processing of 27SB Intermediate into 25S and 5.8S rRNAs

The fact that Nop2p depletion caused a reduction in 25S but not 18S rRNA suggests that the protein might be involved in processing of pre-rRNA. To investigate this possibility, YBH5 (*GAL-NOP2*) cells grown in YPD for 8.5 hours were labeled with [*methyl* -^{3}H] methionine, and chased with an excess of methionine. Since yeast rRNAs are methylated at a large number of sites soon after transcription (Brand et al., 1977), following the labeled methyl groups on the pre-rRNA to the mature rRNAs can reveal the intermediate steps of pre-rRNA processing. In the control L4717 cells, 35S pre-rRNA was almost immediately processed into 27S and 20S pre-rRNAs (Figure 8B). At 4 minutes of chase, most 27S and 20S pre-rRNAs were processed into 25S and 18S rRNAs, respectively. At 8 minutes of chase, almost all of these pre-rRNAs were converted to mature rRNAs (Figure 8B). However, in the Nop2p-depleted cells, 35S and 32S pre-rRNAs significantly accumulated at 2 minutes of chase. Even at 8 minutes of chase, a large amount of 27S pre-rRNA still accumulated with a significant reduction in the formation of 25S rRNA, which is consistent with the finding that the steady-state level of 25S rRNA was reduced after 28 hours of Nop2p depletion. Nevertheless, the processing of 20S to

Figure 8. Depletion of Nop2p results in a defect in pre-rRNA processing.
YBH5(*GAL-NOP2*) (**A**) and L4717 (*NOP2*) (**B**) were shifted from SGal to SD for 8.5 hours, pulse-labeled with [^{3}H-*methyl*]methionine, and chased with excess methionine for 0, 1, 2, 4 and 8 minutes. Total RNA was extracted, separated on an agarose-glyoxal gel, and detected by fluorography. Approximately one-third of the L4717 8-min sample was loaded on the gel.


A
YBH5 (GAL-NOP2)
0 1 2 4 8min.
B
L4717 (NOP2)
0 1 2 4 8min.
35S
32S
27S
25S
20S
18S

18S rRNA in Nop2p depleted cells was similar to that in the control cells (Figure 8A). These results suggest that Nop2p is involved in the processing pathway leading to 25S rRNA.

In this pulse-chase assay, the processing of pre-rRNA was evaluated by following incorporation of label into methyl groups, which had been transferred onto pre-rRNA. Therefore, the possibility had to be excluded that the aberrant processing might be actually a change in the methylation pattern. To do this, [^{3}H]uracil was used to label the backbone of pre-rRNA for pulse-chase analysis. In the control L4717, 35S and 32S precursors were quickly processed (Figure 9B). Chasing of the 27S pre-rRNA to 25S rRNA in the control cells took a longer time than in [*methyl* -^{3}H] labeling. This is possibly because the cell can re-use the labeled uracil resulting from the turnover of RNAs within the cell for a quite long time (Warner, 1991). In YBH5 depleted of Nop2p for 8.5 hours, a significant amount of 35S and 32S precursors were not processed even at 8 minutes of chase (Figure 9A). Compared with the control, a significant delay in the processing of 27S pre-rRNA to 25S rRNA was observed at 8, 16, and 32 minutes of chase, with the formation of 25S rRNA accordingly reduced (Figure 9A). Similar to what was observed in [*methyl* -^{3}H] pulse-chase labeling, the processing of 20S to 18S rRNA was not significantly affected by Nop2p depletion. These results confirm that Nop2p is required for processing of pre-rRNA.

To locate the specific processing step in which Nop2p might be involved, the steady-state levels of pre-rRNAs and rRNAs were estimated by Northern analysis. With probes specific for regions in the ITS1 and ITS2, and in the 18S and 25S rRNAs (Figure 10), the various processing intermediates can be recognized. Consistent with the results from the pulse-chase analysis, Nop2p depletion for 4.5 and 8 hours caused a significant accumulation of the steady-state levels of 35S and 32S precursors, as compared with the non-depletion cells (at 0 time point) and the controls YBH3 and L4717 (Figure 11 A, B, and C). With the probe (oligonucleotide 3) complementary to the 27SA but not 27SB

Figure 9. Pulse-chase labeling analysis of pre-rRNA processing by using [^{3}H]uracil. YBH5 (*GAL-NOP2*) **(A)** and L4717 (*NOP2*) **(B)** were grown in SGal, shifted to SD for 8.5 hours, pulse-labeled with [^{3}H]uracil, and chased with excess uracil for 0, 2, 8, 16, and 32 minutes. The samples were treated with oligo (dT)-cellulose before agarose-glyoxal gel electrophoresis.

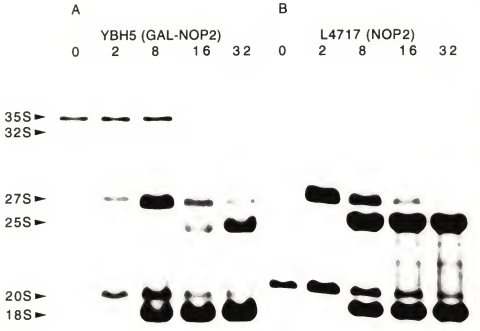

A
YBH5 (GAL-NOP2)
0 2 8 16 32
B
L4717 (NOP2)
0 2 8 16 32
35S
32S
27S
25S
20S
18S

Figure 10. The yeast pre-rRNA processing pathway and the cleavage step requiring Nop2p function. Depletion of Nop2p caused the accumulation of 35S, 32S, 27SA and 27SB pre-rRNAs, but a significant reduction in the production of 25S rRNA. The results indicate that Nop2p functions at the processing step from 27SB to 25S rRNA (as indicated). The oligonucleotides used for Northern blotting (oligo 1, 2, 3, 4 and 5) and RNase protection (oligo 6, 7, and 8) are indicated as short bars below the 35S pre-rRNA.

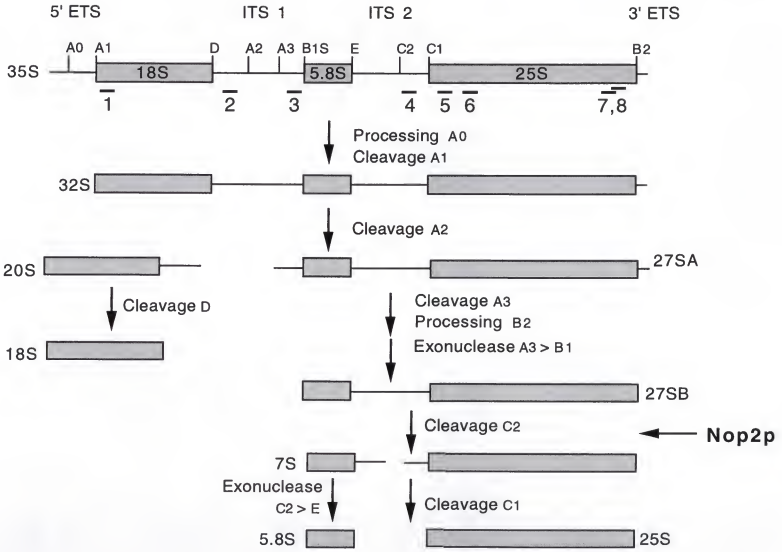
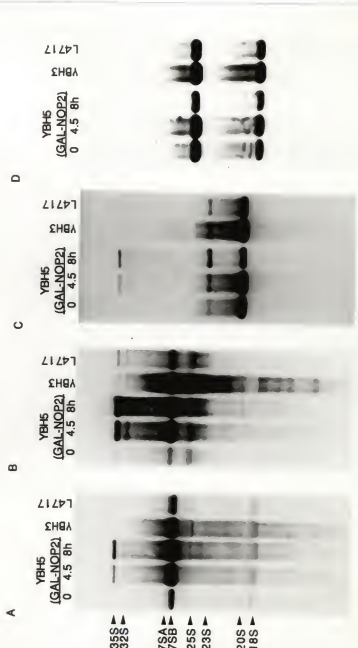

5' ETS
ITS 1
ITS 2
3' ETS
A0
A1
D
A2
A3
B1S
E
C2
C1
B2
35S
18S
5.8S
25S
1
2
3
4
5
6
7,8
Processing A0
Cleavage A1
32S
Cleavage A2
20S
27SA
Cleavage D
18S
Cleavage A3
Processing B2
Exonuclease A3 > B1
27SB
Cleavage C2
Nop2p
7S
Exonuclease
C2 > E
Cleavage C1
5.8S
25S

Figure 11. Northern analysis of steady-state levels of pre-rRNAs and mature rRNAs. Total RNAs were extracted from YBH5 (*GAL-NOP2*) collected at 0, 4.5, and 8 hour points after the medium-shift to YPD, and from YBH3 and L4717 grown in YPD medium. The RNAs were separated on an agarose-glyoxal gel, transferred to a nylon membrane, and probed with ^{32}P-labeled oligonucleotides. (**A**) oligonucleotide 4 (ITS2 probe); (**B**) oligonucleotide 3 (ITS1 probe); (**C**) oligonucleotide 2 (ITS1 probe); (**D**) oligonucleotide 1 (18S probe) and 5 (25S probe) (see Figure 10).

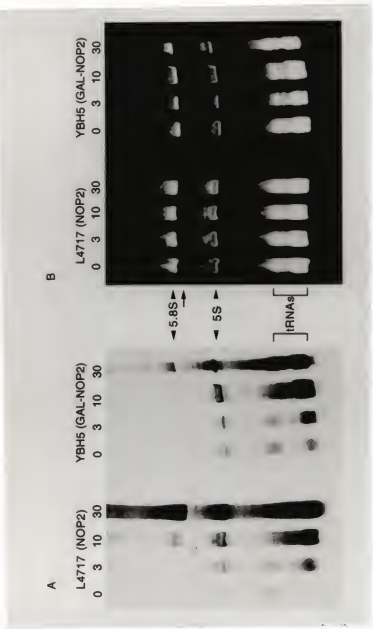

A
YBH5 (GAL-NOP2) 0 4.5 8h
YBH3
L47117
35S
32S
27SA
27SB
25S
23S
20S
18S
B
YBH5 (GAL-NOP2) 0 4.5 8h
YBH3
L47117
C
YBH5 (GAL-NOP2) 0 4.5 8h
YBH3
L47117
D
YBH5 (GAL-NOP2) 0 4.5 8h
YBH3
L47117

pre-rRNA, the accumulation of 27SA pre-rRNA was observed in the Nop2p-depleted cells (Figure 11B). Nop2p depletion also caused a significant accumulation of 27SB pre-rRNA (Figure 11A). The pathway leading 32S precursor to 23S and 27SB intermediates is a minor alternative one in wild type cells (Russell and Tollervey, 1992). Previous studies demonstrated that depletion of Nop1p (Tollervey et al., 1991), or Gar1p (Girard et al., 1992), or snoRNA U3 (Tollervey, 1987) caused the accumulation of 23S. Depletion of Nop2p had no significant effect on the level of this intermediate (Figure 11C). The level of 20S pre-rRNA, a precursor for 18S rRNA, was similar among Nop2p-depleted cells and the control cells (Figure 11C). The sample loading was adjusted according to the levels of 25S and 18S rRNAs (Figure 11D).

The above results indicate that Nop2p functions at the step of processing of 27SB to 25S rRNA (Figure 10). If this is true, then the formation of 5.8S rRNA should also be affected by Nop2p depletion, since 27SB is the common precursor for 25S and 5.8S rRNAs (Figure 10). To test this assumption, the processing of 5.8S rRNA was evaluated by pulse-chase analysis. At 10 and 30 minutes of chase, the formation of 5.8S rRNA was reduced in YBH5 cells depleted of Nop2p for 8.5 hours, as compared with that in the control L4717 (Figure 12A). Approximately equivalent loading was estimated by staining the gel with ethidium bromide (Figure 12B). Since 5S rRNA is transcribed independently by RNA polymerase III, its level should not be affected by the defect in the processing of the large rDNA transcript (Raue and Planta, 1991). A similar rate of the formation of 5S rRNA was observed in YBH5 and the control L4717 cells, suggesting that Nop2p depletion specifically affected the formation of 5.8S rRNA (Figure 12A).

In summary, the results from the pulse-chase analysis and Northern blotting indicate that Nop2p is required for processing of pre-rRNA, specifically at the step of conversion of 27SB pre-rRNA to 25S and 5.8S rRNAs. However, the formation of 18S rRNA from 20S precursor is relatively independent of Nop2p function.

Figure 12. Depletion of Nop2p causes a defect in the synthesis of 5.8S rRNA. (A) YBH5 (*GAL-NOP2*) and L4717 (*NOP2*) were grown in SGal, shifted to SD for 8.5 hours, pulse-labeled with [^{3}H]uracil, chased with excess uracil for 0, 3, 10 and 30 minutes. Total RNAs were extracted, separated on a 12% polyacrylamide-urea gel and detected by fluorography. **(B)** To estimate the loading, the same gel was stained with ethidium bromide. The arrow indicates 0.15-kb RNA standard.


A
L4717 (NOP2)
0 3 10 30
YBH5 (GAL-NOP2)
0 3 10 30
B
L4717 (NOP2)
0 3 10 30
YBH5 (GAL-NOP2)
0 3 10 30
5.8S
5S
tRNAs

Mutational Analysis of Nop2p

Another approach to investigate the function of a protein is to make mutations in the protein. A protein containing point mutations may assume a non-functional conformation at a restrictive condition. If the gene is essential, the mutations will cause a lethal phenotype at a restrictive condition, but not at the permissive condition. At a certain restrictive condition, global changes in cellular processes (cytoskeleton, protein synthesis, replication, transport, etc.) may occur, also causing cell death. However, this possibility can be excluded if the lethal phenotype can be rescued by the gene encoding this protein. One advantage of this approach is that all of the mutated protein generated at a restrictive condition will be non-functional, while it takes a much longer period of time to deplete a protein. Therefore, a deficit in the function will be caused more likely by the mutated protein than by secondary effects. Another advantage is that different alleles of a gene may cause different phenotypes, which helps to reveal multiple functions of the gene product (Huffaker et al., 1987).

Isolation of the Six Temperature-Sensitive *nop2* Alleles

In this study, random point mutations in *NOP2* were generated *in vitro* by using a low fidelity PCR method (Muhlrad et al., 1992). The phenotypes of the cells containing only the mutated *nop2* alleles were analyzed under three restrictive conditions. One is temperature, which has been widely used to isolate conditional mutants (Huffaker et al., 1987; Tollervey et al., 1993). At low (14°C) or high (37°C) temperatures, it may be more demanding for a protein to maintain its functional conformation. Mutation at critical site(s) of the protein can disrupt the functional conformation, causing a mutant phenotype at either high (Ts) or low (Cs) temperatures. Recently, formamide and ethanol were found to be very useful for isolation of conditional mutants (Aguilera and Benitez, 1986; Aguilera,

1994). The chemical groups (C=O, OH, NH) present in formamide and ethanol may compete for noncovalent bonds with the amino acid residues of the protein. Mutation in a protein may weaken the non-covalent bonds formed among the amino acid residues because the amino acid substitution may alter charges or hydrophobicity at the local region. In the presence of formamide or ethanol, the functional conformation of a mutant protein may be disrupted, resulting in either formamide-sensitive (Fs) or ethanol-sensitive (Es) phenotype.

Two mutagenic PCR conditions were tested to generate mutations in the *NOP2*. One includes the addition of 0.15 mM of $MnCl_2$ and a bias in the nucleotide pools. Another includes the bias in the nucleotide pools only. The PCR products and the gapped plasmid pBH49 were cotransformed into YBH3 cells. After plasmid shuffling (see MATERIALS AND METHODS), the effects of the mutations were tested. With the first condition, only very few colonies (an average of 40-50/plate) formed at 25°C (not shown), suggesting that most of the *nop2* alleles were non-functional even under the permissive condition. An estimate of 10,000 colonies carrying the PCR products from the first condition was screened for Ts and Cs phenotypes. However, no conditionally lethal strain was found. With the second condition, an average of 150-200 colonies/plate formed at 25°C. In order to increase the chance of isolating conditionally lethal strains, two additional restrictive conditions, 3% formamide and 6% ethanol, were used. A total of 22,000 colonies carrying the PCR products from the second condition was screened. Some Ts, Cs, and Fs/Ts candidates were isolated, but no Es or Fs only candidate was found (Table 6). To exclude the possibility that the phenotypes might be caused by mutation in other genes on the chromosomes, the plasmids in these candidates were recovered, and their phenotypic effects were tested again in the same genetic background (Table 6). Among the twenty-six Ts candidates, the majority (73%) were caused by the mutation on the chromosomes. No Cs phenotype was found to be plasmid-borne. The plasmid recovered from the Fs/Ts candidate still exhibited the phenotypes (Table 6). To make sure that it was

Table 6. Screening for Conditionally Lethal *nop2* Alleles

Phenotype	Candidates	Plasmid-borne phenotype	ORF-borne phenotype
Ts	26	7	5
Cs	2	0	0
Fs/Ts	1	1	1
Es	0	0	0

Ts: temperature-sensitive. Cs: cold-sensitive. Fs: formamide-sensitive.
Es: ethanol-sensitive. ORF: open reading frame.

the mutation in the protein that caused the phenotypes, the open reading frame (ORF) of the alleles was subcloned and its phenotypic effect was tested again. A total of six conditionally lethal alleles were isolated. All six alleles conferred the cells with the temperature sensitive phenotype, while they still supported cell growth to some degrees at the permissive temperature (25°C) or at 30°C (Figure 13). In liquid medium, 8 hours after the temperature shift, the growth curve of the six Ts strains already reached a plateau, while the wild type L4717 still grew in log-phase (Figure 14). Interestingly, *nop2-10* strain expressed not only Ts, but also Fs at 25°C (Figure 15, *nop2-10*), which suggests that the mutation at an additional site(s) of this allele caused this additional phenotype that was not expressed in the other Ts strains. The conditionally lethal phenotype of the six Ts strains was rescued by another plasmid carrying the wild type *NOP2* gene (Figure 15 and not shown). This confirms that the phenotype was caused by mutation in *NOP2*.

To exclude the possibility that the phenotype might be due to rapid protein degradation caused by the mutations, immunoblotting analysis of the mutated versions of the protein was performed. After 6 hours at 37°C , all of the six mutated versions of the protein were present, though the amounts were relatively lower than the two controls (L4717 and YBH3) (Figure 16). This result suggests that the mutant proteins were relatively stable. Compared with the two controls (L4717 and YBH3), there was no change in protein mobility of *nop2-3* and *nop2-4*. However, *nop2-5* protein moved faster than the other mutants and the controls (Figure 16). Sequencing of this allele revealed that there was an amber mutation near the 3' end of the ORF, causing a truncation of 14 amino acids at the C terminus (see Figure 19). There was also a change in the protein mobility of *nop2-6*, *nop2-9* and *nop2-10* (Figure 16), which may be due to changes in charge or modification of the mutant proteins.

The above results indicate that the phenotypes of the six *nop2* alleles isolated are due to the mutations in the ORF of the gene and that the amino acid substitutions in the mutant proteins result in disruption of the functional conformation of the protein at the

Figure 13. The effect of temperature on the growth of the six *nop2* temperature-sensitive (ts) strains. Serial five-fold dilutions of the cells of L4717, YBH3, and the six *nop2* ts strains (*nop2-3, nop2-4*, *nop2-5*, *nop2-6*, *nop2-9* and *nop2-10*) were grown on YPD plates at 25°C, 30°C or 37°C.

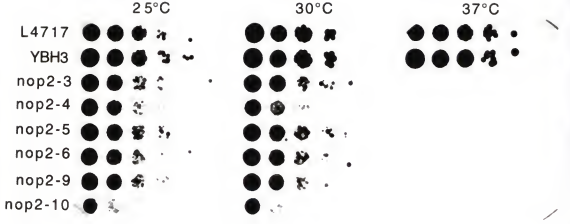

25°C
30°C
37°C
L4717
YBH3
nop2-3
nop2-4
nop2-5
nop2-6
nop2-9
nop2-10

Figure 14. Growth curves of the six *nop2* ts strains. The cells of L4717 and the six *nop2* strains (*nop2-3*, *nop2-4*, *nop2-5*, *nop2-6*, *nop2-9* and *nop2-10*) were grown in YPD at 25°C and shifted to 37°C (at 0 time). At the indicated times, the cell densities were measured (A_{600}).

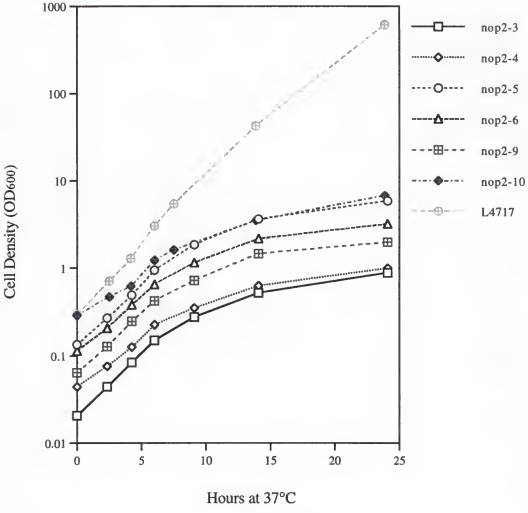
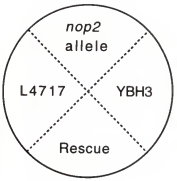

1000
100
10
1
0.1
0.01
Cell Density (OD600)
0
5
10
15
20
25
Hours at 37°C
nop2-3
nop2-4
nop2-5
nop2-6
nop2-9
nop2-10
L4717

Figure 15. Mutations in *NOP2* caused temperature sensitive and formamide sensitive phenotypes. The cells of *nop2-9* and *nop2-10*, and the corresponding "rescue" strains (carrying pJPA29 plasmid) were streaked on YPD plates with or without 3% formamide, and incubated either at the restrictive (37°C) or the permissive (25°C) temperature as indicated. The growth of wild type L4717, and YBH3 (carrying plasmid-borne *NOP2*) are shown as controls.

nop2-9

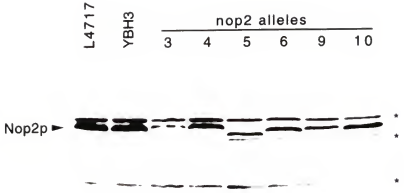


37°C 25°C

nop2-10

25°C - FA 25°C + FA 37°C - FA

Figure 16. Mutant *nop2* proteins are relatively stable. The cells of L4717, YBH3 and the six *nop2* ts strains (*nop2-3*, *nop2-4*, *nop2-5*, *nop2-6*, *nop2-9* and *nop2-10*) were grown in liquid YPD at 37°C for 6 hours. Total cellular proteins were extracted and analyzed by Western blotting with a polyclonal antibody to Nop2p. The position of Nop2p is indicated. Non-specific bands are labeled with stars.


L4717
YBH3
nop2 alleles
3
4
5
6
9
10
Nop2p

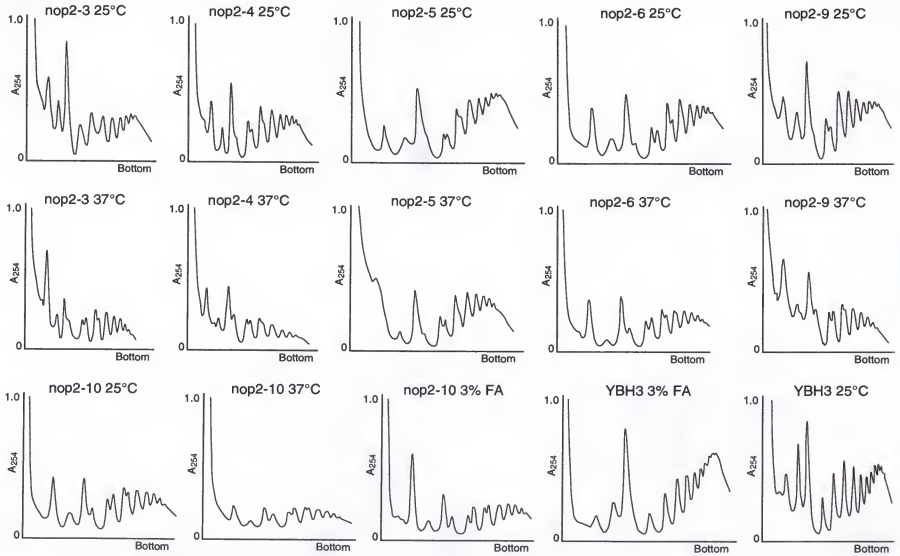
restrictive conditions. Since *nop2-10* allele caused Fs, in addition to Ts phenotype, several structural domains may exist in the protein. Mutation in some domains may result in Ts phenotype only, as was shown in the other five *nop2* alleles, while mutation in other domains may cause Fs or both Ts and Fs phenotypes. The Ts or Ts/Fs phenotypes are most possibly caused by the direct effects of mutation in *NOP2* gene, though it is also possible that mutation in *NOP2* may make the cells extremely "sick" and that a second "hit" with heat or formamide may actually cause cell death.

Characterization of the Six *nop2* Alleles

One advantage of using a mutational analysis approach is that it can reveal multiple functional domains in a protein. Since each of the six *nop2* alleles contains multiple point mutations, analysis of the effects of these different mutations would help understand more about the protein's function.

Each allele was analyzed for its effect on the synthesis of ribosomes. The six Ts strains and the control YBH3 strain (carrying plasmid-borne *NOP2*) were incubated at 37°C for two hours, and the levels of ribosomal subunits and polysomes were measured. In the control YBH3, incubation at 37°C did not cause any significant change in the polysome profile (not shown). In contrast, even at the permissive temperature (25°C), all of the Ts strains, except *nop2-3*, already exhibited a significant change in the polysome profile, including a reduction in the peaks of the 60S subunit and the 80S monosome, a relative accumulation of 40S subunit, and appearance of halfmer polysomes (Figure 17). At 37°C for 2 hours, a more severely abnormal polysome profile was seen in all of the six Ts strains (Figure 17). However, the degree of the severity in the defect appeared to vary among the six Ts strains, with *nop2-10* strain affected most. When YBH3 was incubated in the medium containing 3% formamide at 25°C for two hours, the peaks of both 40S and 60S subunits were proportionally reduced. However, there was no change in the peaks of 80S

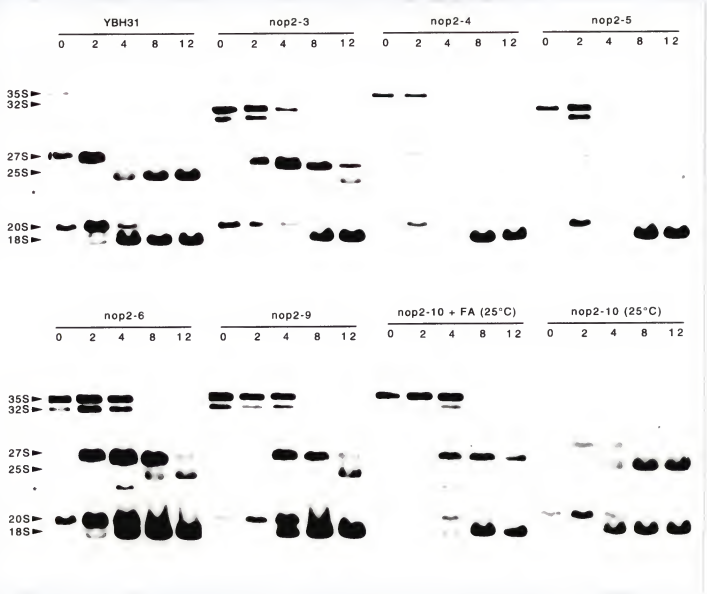
Figure 17. Mutation in *NOP2* impairs the synthesis of 60S ribosomal subunit. The six *nop2* ts strains (*nop2-3*, *nop2-4*, *nop2-5*, *nop2-6*, *nop2-9* and *nop2-10*) were grown in YPD at the restrictive (37°C) or permissive (25°C) temperature for 2 hours. nop2-10 and YBH3 were grown in YPD containing 3% formamide at 25°C for 2 hours. YBH3 was grown at 25°C. Cell lysates were analyzed on sucrose density gradients. The ribosome subunits, monoribosome and polyribosomes were measured as A_{254} values.


nop2-3 25°C
nop2-4 25°C
nop2-5 25°C
nop2-6 25°C
nop2-9 25°C
nop2-3 37°C
nop2-4 37°C
nop2-5 37°C
nop2-6 37°C
nop2-9 37°C
nop2-10 25°C
nop2-10 37°C
nop2-10 3% FA
YBH3 3% FA
YBH3 25°C
1.0
0
A254
Bottom

monomer and polysomes, and no halfmer polysome was seen (Figure 17). When incubated in the medium containing 3% FA at 25°C, only *nop2-10* strain showed a similar abnormality that was seen when incubated at 37°C (Figure 17 and not shown). The results confirm that Nop2p is required for the synthesis of 60S ribosomal subunit. Since there are different degrees of severity in the defect caused by these six alleles, and only *nop2-10* strain showed the formamide-sensitive phenotype, it is possible that several regions within Nop2p may be required for the functional conformation.

The effects of the six Ts alleles on pre-rRNA processing were evaluated by pulse-chase labeling analysis. After 2 hours at 37°C, all of the six Ts strains (*nop2-10* not shown) exhibited a significant accumulation of 35S and 32S precursors. At the same time, the formation of 25S rRNA was significantly delayed and reduced (Figure 18). In the control YBH31 (carrying plasmid-borne *NOP2*) grown under the same conditions, the processing of pre-rRNA was rapidly completed (Figure 18). There was no accumulation of 20S pre-rRNA and the formation of 18S rRNA was not significantly affected in the six Ts strains at 37°C (Figure 18). When grown in 3% formamide medium at 25°C, *nop2-10* strain also exhibited a similar defect in the processing that was observed at 37°C (Figure 18). However, when grown in the medium without 3% formamide at 25°C, the processing in *nop2-10* appeared to be similar to that in the control YBH31 (Figure 18). These results are consistent with those from Nop2p depletion analysis. However, the 27S processing intermediate rapidly disappeared in *nop2-4* and *nop2-5* strains, though the formation of 25S rRNA in these two strains was also reduced (Figure 18). In all of the other Ts strains, accumulation of this intermediate was significant, even at 8 minutes of chase (Figure 18). Since Nop2p was involved in the methylation of only one site on 25S rRNA sequence (see below), It is most possible that the 27S intermediate in *nop2-4* and *nop2-5* strains was rapidly degraded. These results confirm that Nop2p is required for the processing pathway leading to 25S rRNA. The fact that 27S processing intermediate did not accumulate in *nop2-4* and *nop2-5* strains suggests that the region(s) mutated in *nop2-4* and *nop2-5* alleles

Figure 18. Mutation in *NOP2* causes a defect in pre-rRNA processing. YBH31 (carrying plasmid-borne *NOP2*), *nop2-3*, *nop2-4*, *nop2-5*, *nop2-6* and *nop2-9* were grown in SD medium, and collected after temperature shift to 37°C for two hours. *nop2-10* was grown in SD medium with or without 3% formamide at 25°C for two hours. The cells were pulse-labeled with [*methyl*-^{3}H] methionine, and chased with excess methionine for 0, 2, 4, 8 and 12 minutes. Total RNA was separated on agarose-glyoxal gel, and detected by fluorography. The star indicates the position of 23S pre-rRNA.


YBH31
nop2-3
nop2-4
nop2-5
0 2 4 8 12
35S
32S
27S
25S
20S
18S
nop2-6
nop2-9
nop2-10 + FA (25°C)
nop2-10 (25°C)

was required for the stability of 27S intermediate. It is possible that certain region(s) in Nop2p may be involved in the association of other proteins or snoRNPs with 27S intermediate and that disruption of such association may subject 27S intermediate to a rapid degradation.

Structure and Function Analysis of Nop2p

Analysis of the sites of point mutations in a protein can reveal the relationship among the protein primary sequence, conformation and function. To determine the changes in the primary structure of the mutant Nop2p, all of the six alleles were sequenced, and mutation sites were located (Table 7). Most of the point mutations caused amino acid substitutions (missense mutations) (Figure 19), while some mutations did not change amino acid residues (silent mutations) (Table 7). Only one point mutation resulted in a non-sense mutation (K_{605} to amber mutation in *nop2-5*) (Figure 19). Muhlrad et al. reported that the most common base changes are A/T>G/C transitions (Muhlrad et al., 1992). In this study, A/T>G/C and G/C>A/T transitions comprised 57% of the total base changes in the six *nop2* alleles, with G/C>A/T the most common change (33%) (Table 8). Each of the six alleles contained from 7 to 12 missense mutations. In *nop2-10*, all of seven point mutations were located in the C-terminal half of the protein sequence (from aa 254 to aa 560). In contrast, the mutations in all the other five alleles spanned the whole protein sequence. According to the sequencing data, replacement of some amino acid residues caused changes in charge or polarity at these sites. For example, $I_{309}K$ change in *nop2-3* made this site become positively charged (Figure 19). Protein secondary and tertiary structures depend on the non-covalent interactions among the side chains of amino acid residues. Therefore, it is likely that the amino acid substitutions in the mutant Nop2p make the functional conformation of the protein unstable or disrupted at the restrictive conditions.

Table 7. PCR-Generated Point Mutations in *NOP2*

Alleles	Mutations†
nop2-3	A_{145}>G, T_{197}>A*, G_{531}>A, A_{778}>G, T_{902}>A*, T_{925}>A, T_{940}>C*, A_{1103}>T, C_{1202}>T*, G_{1291}>A, G_{1408}>A, A_{1640}>C
nop2-4	G_{207}>A, A_{252}>G, A_{256}>T, A_{286}>T, G_{427}>T, G_{520}>A, T_{729}>C, A_{829}>G, C_{839}>T*, G_{915}>A*, T_{1045}>C, G_{1131}>A, C_{1489}>T
nop2-5	G_{192}>A, G_{561}>A, A_{826}>G, C_{1062}>A, T_{1193}>C*, G_{1131}>A, A_{1396}>T, G_{1647}>A, A_{1749}>C, G_{1758}>T, A_{1812}>T#
nop2-6	A_{42}>T, A_{322}>T, G_{437}>A, G_{465}>A, T_{594}>A*, A_{617}>G*, T_{720}>A, T_{792}>A, CA_{944}>TT, C_{1005}>T*, T_{1319}>A, A_{1450}>T, C_{1547}>T*, G_{1718}>T*
nop2-9	A_{86}>G*, AA_{247}>TT, C_{350}>T*, T_{368}>G, A_{450}>G, T_{473}>A*, G_{504}>A, T_{520}>C, C_{1162}>A, G_{1174}>A, C_{1347}>T, A_{1529}>T
nop2-10	T_{692}>C*, A_{725}>T*, T_{759}>A, G_{898}>A, C_{943}>A, G_{972}>T, A_{1107}>T, T_{1292}>C*, G_{1494}>A, C_{1675}>A

† Numbers indicate positions of missense mutations in *NOP2* sequence.

* Silent mutations

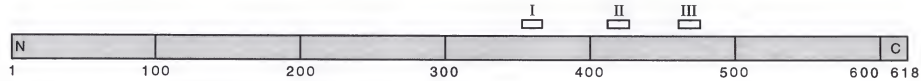
Nonsense mutation

Table 8. Types of Base Changes in the Six *nop2* Alleles

Type of base change	% observed
Transitions	
A/T>G/C	24%
G/C>A/T	33%
Transversions	
A/T>T/A	29.3%
A/T>C/G	0.26%
G/C>T/A	11.6%
G/C>C/G	0

Figure 19. Amino acid substitutions in *nop2* mutants. Nop2p with a total of 618 amino acid residues is shown as a box from residue 1 (N-terminus) to residue 618 (C-terminus). The amino acid changes (indicated with arrows) and the corresponding positions in the Nop2p are shown for the six *nop2* alleles (*nop2-3*, *nop2-4*, *nop2-5*, *nop2-6*, *nop2-9*, *nop2-10*). Amb: amber mutation. Motif I (SAM binding domain): from residue 347 to 363; motif II: from residue 415 to 430; motif III: from residue 468 to 484.

Summary of Amino Acid Changes in nop2 Mutants



nop2-3

K R49 A T178 E G260 I K309 V A314 K N368 G D431 G D470 Q H547 I T549

nop2-4

D N70 R G85 K I86 E V108 A S143 M I174 F L244 K R279 G S306 I T349 A T378 P L497

nop2-5

E K65 E K188 N S276 A D355 A T378 N I466 G S550 T A584 D Y587 K605 Amb

nop2-6

T S15 E G108 M I146 E K156 S T199 Y N241 F I263 P L315 E V484 L F573

nop2-9

K L83 N K123 K E151 V M169 M T174 A D388 R H392 P L449 V M482 K N510

nop2-10 (ts and fs)

S T254 G S300 P Q315 G C325 T S370 V I499 A E560

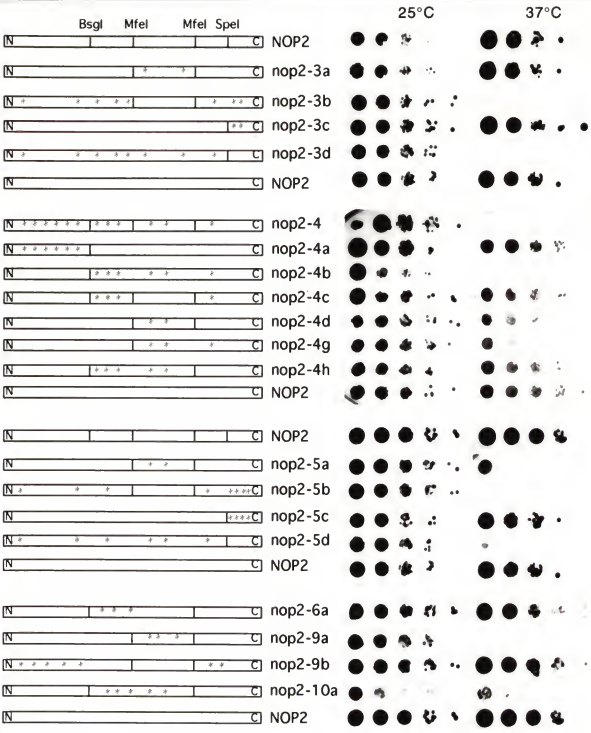
Since the random mutagenesis PCR method was used in this study, it was predictable that multiple mutations spanning the whole protein sequence would occur. This was confirmed by the results from sequencing of the six *nop2* alleles (Table 7). In many cases, Ts phenotype is caused by one or a few point mutations at critical sites ("hot spots"). To determine the mutations responsible for the phenotype, several restriction sites within the ORF of each *nop2* allele was used to separate the mutations in each allele into several groups. The phenotypic effects of each group or the combination of several groups of mutations were analyzed.

***nop2-3* allele.** In this allele, the two point mutations within *Mfe*I fragment are not located in any of the motifs (Figure 19), and did not cause the phenotype (Figure 20, nop2-3a). Another two point mutations located 3' to *SpeI* site were not required for the phenotype (Figure 20, nop2-3c).

***nop2-4* allele.** In this allele, one *Bsg*I and two *Mfe*I sites were used to separate the allele into four parts. The effect of the mutations in each part was tested. The six point mutations upstream to *Bsg*I site did not cause any detectable phenotype at 37°C (Figure 20, nop2-4a). However, the six point mutations downstream to *Bsg*I site caused the Ts phenotype (Figure 20, nop2-4b). These six point mutations were then separated into three groups. Each group independently or the combination of the two groups did not cause the Ts phenotype (Figure 20, nop2-4c, nop2-4d, nop2-4g and nop2-4h). However, the effect of each group seemed to be additive (Figure 20, nop2-4g). The mutation in the SAM binding domain ($I_{304}T$) (Figure 19) with another mutation in the *Mfe*I fragment did not cause the Ts phenotype (Figure 20, nop2-4d). The results suggest that at least the three point mutations located in the three regions of Nop2p are required to cause the phenotype. Mutation in one of them may not significantly change the conformation.

***nop2-5* allele.** The two point mutations in *Mfe*I fragment of this allele caused the phenotype, though this allele was a little leaky (Figure 20, nop2-5a). Since $A_{378}T$ also occurs in *nop2-4* , but did not cause Ts phenotype, it may be A_{355} to D change that caused

Figure 20. Mapping of the mutations in the six *nop2* alleles. The point mutations (labeled as asterisk in boxes) in each of the six *nop2* alleles were separated into groups using the restriction sites *Bsg*I, *Mfe*I, or *Spe*I (see METHODS AND MATERIALS for detailed descriptions). The *nop2* alleles thus constructed are shown as the boxes (the left panel). Serial dilutions of the cells carrying the corresponding *nop2* alleles were grown on the SD plates at 25°C (the middle panel) or 37°C (the right panel). YBH31 strain carrying the plasmid-borne *NOP2* (labeled as NOP2) is shown as a control.


BsgI
MfeI
MfeI
SpeI
25°C
37°C
N
C
NOP2
nop2-3a
nop2-3b
nop2-3c
nop2-3d
NOP2
nop2-4
nop2-4a
nop2-4b
nop2-4c
nop2-4d
nop2-4g
nop2-4h
NOP2
NOP2
nop2-5a
nop2-5b
nop2-5c
nop2-5d
NOP2
nop2-6a
nop2-9a
nop2-9b
nop2-10a
NOP2

the Ts phenotype (Figure 19). This change makes this site negatively charged, which is located in the SAM binding domain (Figure 19). However, it is not clear if the phenotype was caused by inactivation of the methylase activity or by disruption of the functional conformation. However, without these two point mutations, the other point mutations in this allele also conferred the cells with the Ts phenotype (Figure 20, nop2-5b and nop2-5d), suggesting that at least two structural domains present in Nop2p and that the mutation in either domain causes disruption of the functional conformation. Truncation of 14 amino acid residues at the C terminus caused by a nonsense mutation (K_{605} to amber mutation) did not significantly affect the function (Figure 20, nop2-5c). The three point mutations downstream to *Spe*I site did not cause Ts phenotype (Figure 20, nop2-5c).

***nop2-6* allele.** One *Mfe*I site was lost by mutation in this allele. The three point mutations in the *BsgI*/*Mfe*I fragment were not responsible for Ts phenotype (Figure 20, nop2-6a).

***nop2-9* allele.** In this allele, the three point mutations within the *Mfe*I fragment caused the phenotype (Figure 20, nop2-9a). However, all of the three sites are outside the conserved motifs (Figure 19). The other point mutations in this allele did not exhibit any phenotype (Figure 20, nop2-9b).

***nop2-10* allele.** Only the five point mutations in *BsgI*/*Mfe*I fragment were responsible for the Ts phenotype (Figure 20, nop2-10a). This allele caused both Ts and Fs phenotypes. It is not known if the two phenotypes can be separated. However, it is possible that at least two structural domains exist in *BsgI*/*Mfe*I fragment. Mutation in one domain may cause one phenotype but not the other.

In summary, there is no "hot spot" mutation site in Nop2p. The amino acid substitutions in the six alleles are spread over the whole protein sequence. The $A_{355}D$ change alone (in *nop2-5*) seems to cause a "leaky" Ts phenotype. However, in all of the other alleles, at least three point mutations are required for causing the Ts phenotype. This suggests that there may be several structural domains within Nop2p. Mutation in more

than one structural domains will cause a significant conformation change under the restrictive conditions.

Nop2p and Methylation

Nop2p Contains A Putative SAM Binding Domain

A further study was done in order to understand the detailed mechanism by which Nop2p is required for pre-rRNA processing. Proteins with their functions essential for cell viability (encoded by "house-keeping" genes) usually contain evolutionarily conserved sequences (domains or motifs). Therefore, analysis of the primary structure of a protein will give a clue to its function. Koonin found that human p120 protein as well as *E. coli.* Fmuv protein contains the consensus sequence, the SAM binding domain, which is conserved in a broad spectrum of methylases that modify proteins, nucleic acids, and lipid substrates (Koonin, 1994). The function of Fmuv is not known, but it is encoded in the same operon with fmt, the Met-tRNAfMet transformylase (Meinnel et al., 1993). Three other motifs are also present in p120 and Fmuv proteins. Since Nop2p is highly homologous to the p120 protein (de Beus et al., 1994), the possibility that Nop2p might contain SAM binding domain was investigated. Sequence alignments revealed that Nop2p also contained the SAM binding domain and the other two motifs (Figure 21). This result suggests that Nop2p may have methylase activity. Since Nop2p is required for processing of 27SB to 25S rRNA, it is possible that Nop2p might be involved in the methylation at the 27S level (late methylation), and that the methylation might be required for a correct secondary structure of 27S pre-rRNA for processing. Among the methylations in 25S rRNA sequence, only four groups are added as late methylation (at 27S level) (Brand et al., 1977). Two of them are contained in a stretch of sequence ($UmGm\Psi UC_{2922}$), which is evolutionarily conserved from bacteria to human (Table 9).

Table 9. Conserved UmGmΨUC in the Large Subunit rRNA Sequences

Species	LSU rRNA	Sequence and site of ribose methylation	Total number of ribose methylations
E.coli	23S	C_{2551}UmGUUCG	3
Yeast (Mito)	21S	C_{2790}UmGUUCG	2
Yeast	25S	U_{2917}UmGmΨUCA	37
Xenopus	28S	U_{3624}UmGmΨUCA	70
Human	28S	U_{4457}UmGmΨUCA	70

LSU: large subunit; Mito: mitochondria; Ψ: pseudouridine.

Figure 21. Multiple sequence alignment of yeast Nop2p, human p120, and *E. coli* Fmuv proteins. The alignment was generated by using MacDNASIS software. Amino acid numbers are indicated in parentheses. The positions of the SAM-binding motif, the motif 2 and motif 3 are indicated. Con: consensus sequence.

```
                                        Motif 1
                                    ┌─ SAM-Binding ─┐
Nop2   (330)  QAASSFLPVIALDPHENERILDMAAAPGGKTTYISAMMKNTGCVFANDANKS
P120   (369)  QGASSMLPVMALAPQEHERILDMCCAPGGKTSYMAQLMKNTGVILANDANAE
Fmuv   (231)  QDASAQGCMTWLAPQNGEHILDLCAAPGGKTTHILEVA-PEAQVVAVDIDEQ

Con           Q. AS. . . . ◻. . L. P. . . E. ILD◻. . APGGKT. . ◻. . ◻◻. . . . . ◻◻A. D◻. . .


                                                ┌── Motif 2 ──┐
Nop2   RTKSLIANIHRLGCTNTIVCNYDAR-EFPKVIGGFDRILLDAPCSGTGVIGKDQSVKV
P120   RLKSVVGNLHRLGVTNTIISHYDGR-QFPKVVGGFDRVLLDAPCSGTGVISKDPAVKT
Fmuv   RLSRVYDNLKRLGMKATVKQGDGRYPSQWCGEQQFDRILLDAPCSATGVIRRHPDIKW

Con    R. . . ◻. . N◻. RLG. . . T◻. . . . . FDR◻LLDAPCS. TGVI. +. . . ◻K.


                                                ┌── Motif 3 ──┐
Nop2   SRTEKDFIQIPHLQKQLLLSAIDSVDCNSKHGGVIVYSTCSVAVEEDEAVI  (489)
P120   NKDEKDILRCAHLQKELLLSAIDSVNATSKTGGYLVYCTCSITVEENEWVV  (528)
Fmuv   LRRDRDIPELAQLQSEIL----DAIWPHLKTGGTLVYATCSVLPEENSPAD  (386)

Con    . +. -+D◻. . . . . LQ. . ◻L      D. ◻. . . . K. GG. ◻VY. TCS◻. . EE. . . ◻◻


       ◻ = A, F, I, L, M, V              + = K, R          - = D, E
```

Level of Methylation at the Conserved UmGmΨUC$_{2922}$ Site was Reduced during Nop2p Depletion

To investigate if Nop2p was involved in the methylation at the conserved UmGmΨUC$_{2922}$ site, an RNase protection assay was designed to detect any effect of Nop2p depletion on the methylation at this site. The cellular RNAs in YBH5 cells depleted of Nop2p for 8.5 hours and the wild type cells (L4717) were first pulse labeled with [*methyl*-^{3}H]methionine and chased with an excess of methionine. Oligonucleotide (oligo) 7 was used to protect the UmGmΨUC$_{2922}$ site (Figure 22A). Oligo 8, used as a control, would protect two early methylation sites in the sequence immediately following that protected by oligo 7 (Figure 22A). Oligo 6, used as an internal reference control, would protect the three early methylation sites located at a significant distance from the UmGmΨUC$_{2922}$ site.

In Nop2p-depleted cells, the intensity of the band protected by oligo 7 was significantly reduced compared with that in the wild type L4717 cells (Figure 22B). To evaluate the efficiency of isotope incorporation, equivalent amounts of the labeled RNAs from L4717 and from the Nop2p-depleted (YBH5) cells were partially digested with RNaseT1. Nop2p-depletion caused a reduction in the incorporation of [*methyl*-^{3}H]methionine label into the RNAs (Figure 22C). Even considering this factor, comparison of the intensities of the bands protected by oligo 7 and oligo 8 (both covering two methylation sites) in YBH5 revealed that the intensity of the band protected by oligo 7 was reduced to a much greater degree (Figure 22B). To correct for this difference in the efficiency of isotope incorporation, the radioactivity in the protected bands was measured by scintillation counting. The data from YBH5 were normalized by using the amount of radioactivity protected by the control oligo 6. In three independent experiments, the oligo 7-protected signal from YBH5 was only 66% (±3%) of the control signal from L4717. In

Figure 22. Nop2p depletion leads to under-methylation at the UmGmΨUC site in 25S rRNA sequence. (A) The positions of the oligonucleotides 6, 7 and 8 complementary to 25S rRNA: oligonucleotide 6 complementary to the stretch from nucleotide 635 to 674 (sequence not shown); oligonucleotide 7 to the boxed sequence; oligonucleotide 8 to the shaded sequence. **(B)** RNase protection assay. L4717 (*NOP2*) and YBH5 (*GAL-NOP2*) were grown in SD medium for 8.5 hours, and pulse-labeled with [*methyl*-^{3}H] methionine for 3 minutes. After hybridization with the oligonucleotides, the labeled RNA was digested with RNase A and T_1. The protected fragments were resolved in a 12% polyacrylamide-urea gel, and detected with fluorography. **(C)** Equivalent amount of the labeled RNA samples used for RNase protection were partially digested with RNaseT_1 and electrophoresed on the same gel. **(D)** To estimate the total RNA used for RNase protection, the same samples were separated on the gel and stained with ethidium bromide (EtBr).

A
B
C
D
25S rRNA
1
3392
6
8
7
2861
2975
Oligonucleotide
6
8
7
L4717
Y8H5
RNase T1 (0.1)
EtBr

Table 10. RNase Protection of [^{3}H]-Methylated rRNA.

	Oligo 6 Protection*			Oligo 7 Protection			Oligo 8 Protection		
Experiment	L4717 DPM	Factor	%	L4717 DPM	YBH5 DPM	%	L4717 DPM	YBH5 DPM	%
1	3929	2.57	100	2163	1522	70	3124	3058	98
2	1423	3.27	100	723	471	65	716	699	98
3	975	2.19	100	334	214	64	325	307	95
Mean						66.3			97.0
Standard Deviation (S_{n-1})						±3.2			±1.7

*Oligonucleotide 6 was used as an internal standard. YBH5 DPM data for each experiment were multiplied by a correction factor in order to normalize to 100%.

contrast, the signal of oligo 8-protected band from YBH5 was 97% (±1.7%) of that from L4717 (Table 10).

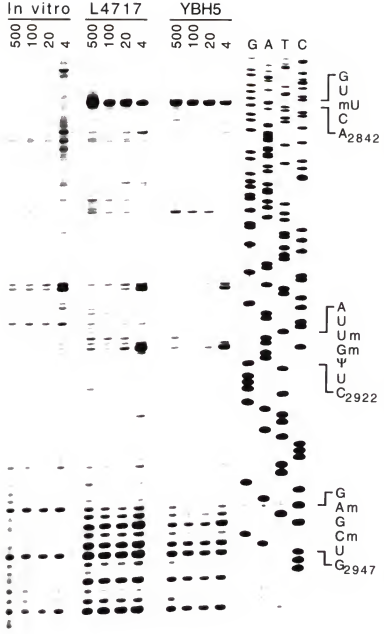
There are two explanations for the 33%, but not 100%, reduction of methylation at $UmGm\Psi UC_{2922}$ site. One is that Nop2p may be responsible for only one of the two methylations. Another explanation is that another methylase may also modify these sites, but with less efficiency.

In summary, the methylation at $UmGm\Psi UC_{2922}$ site was significantly reduced during Nop2p depletion. However, it is not clear about the relationship between the reduced methylation and the defective processing, both of which occurred almost at the same time during Nop2p depletion. They may be only temporally related or may have a cause-effect relationship.

Analysis of Methylation by Using Primer Extension Assay

To understand more about the relationship between methylation and Nop2p function, primer extension analysis was used. It was found that during reverse transcription, reverse transcriptase may "pause" at a site modified by 2'-O-methylation on an RNA template if the concentration of dNTPs are low (concentration dependent pause) (Maden et al., 1995). To test this technique, RNAs from the L4717 cells and the *in vitro* 27S rDNA transcript (with no modification) were used for primer extension. Concentration dependent pauses at the sites modified by base methylation (mU_{2840}) and 2-O'-methylation ($UmGm_{2919}$ and $AmGCm_{2945}$) were observed with the RNAs from L4717, but not with the *in vitro* transcript (Figure 23). This result indicates that with this assay, a 2'-O-methylated site or a base methylated site can be distinguished from a non-methylated one. A few pauses were also seen with the *in vitro* transcript (Figure 23), which may be caused by RNA secondary structures. To evaluate the effect of Nop2p depletion on rRNA methylation, total RNAs from the cells depleted of Nop2p for 8.5 hours

Figure 23. Analysis of rRNA methylation by using concentration dependent pause assay. The RNAs from L4717 and YBH5 cells grown in YPD medium for 8.5 hours were used as templates. *In vitro* 27S rDNA transcript was included as a negative control. The templates were mixed with a primer JA22, which was extended in the presence of [α-^{35}S]dATP by reverse transcriptase to the methylation sites as indicated. Serial five-fold reductions in dNTP concentrations (500 to 4 μM) for extension are indicated.


In vitro
L4717
YBH5
500
100
20
4
G A T C
G
U
mU
C
A2842
A
U
Um
Gm
Ψ
U
C2922
G
Am
G
Cm
U
G2947

were subject to primer extension analysis with a primer that is extended from G_{2969} to $UmGm\Psi UC_{2922}$. However, a pause was still observed at $UmGm\Psi UC_{2922}$ site after Nop2p depletion, which suggests that the site was still modified (Figure 23). The mU_{2840} and $AmGCm_{2945}$ sites were also modified during Nop2p depletion (Figure 23).

The effect of the mutation in *nop2* on the methylation was also analyzed. Two Ts strains were tested. *nop2-4*, with a tight Ts phenotype, was incubated at 37°C for 2 hours, the time point when an obviously abnormal polysome profile was already observed (see Figure 17). *nop2-6,* with a leaky Ts phenotype, was also tested with 2 or 24 hours of incubation at 37°C. However, in both strains under the restrictive condition, the concentration dependent pause bands were seen with no difference from the control YBH31 (not shown). Considering the possibility that Nop2p might be involved in methylation other than the $UmGm\Psi UC_{2922}$ site, more primers were used to analyze all of the mapped methylation sites on 25S rRNA sequence (Table 11). Nevertheless, no detectable reduction of methylation was seen in the two Ts strains at the restrictive condition (not shown).

One explanation for this result is that Nop2p might not be involved in methylation, which is inconsistent with the result from RNase protection experiment. Another one is that a slow methylation at $UmGm\Psi UC_{2922}$ site in Nop2p depletion or Ts cells still occurred and that mature rRNAs have a long half-life, so that in a mixture of methylated and non-methylated RNA templates, the difference could not be differentiated by concentration dependent assay. One possible solution to this problem is to use 27S intermediate, instead of total RNAs, for primer extension. However, because of very low level of 27S and no 27S band visible on the gel stained with ethidium bromide, efforts to gel-isolate 27S for primer extension were made twice without success.

Table 11. Methylation Sites of 25S rRNA and Primers Used for Concentration Dependent Pause Assay

Methylation sites	Primers*
mA_{643}, $AmCm_{648}$	E
$GmAAm_{805}$, Am_{815}, Gm_{865}, Um_{896}	D
Am_{1131}	C
Cm_{1435}, $AmGm_{1448}$	B
Um_{1885}	A
mA_{2140}, Cm_{2195}, Am_{2217}, Am_{2254}, $mCAAmAm_{2279}$	JA31
Um_{2415}, mU_{2632}, Am_{2638}	JA30
Um_{2725}, $GmAGm_{2788}$, mU_{2838}, $UmGm_{2919}$, $AmGCm_{2943}$	JA22

*: see Table 4 for the primer sequences.

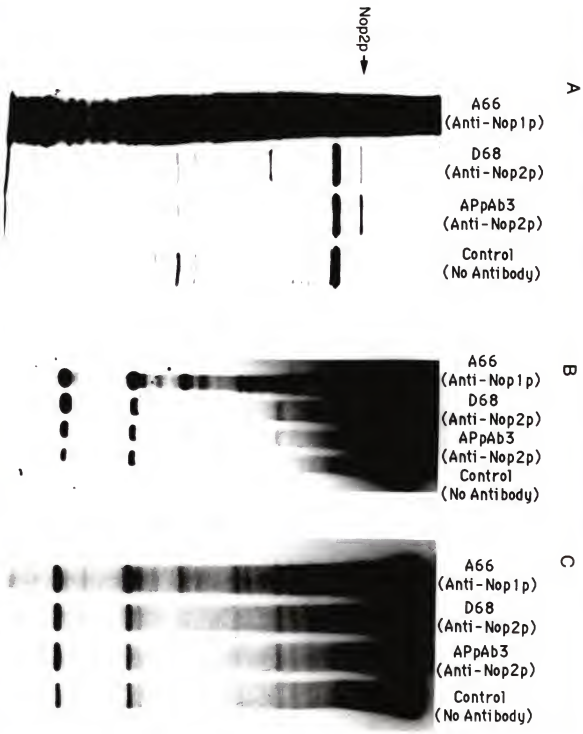
Interaction of Nop2p with Other Macromolecules

Immunoprecipitation Approach

Recent studies suggest that certain nucleolar proteins are associated with snoRNAs as a functional complex (Fournier and Maxwell, 1993). Many yeast nucleolar proteins identified so far contain recognizable RNA binding motifs, such as RRM, GAR domain. Nop2p does not contain any of the known RNA binding motifs. However, in several cases, proteins without these motifs were also found to be associated RNA. One example is the L1 protein, a component of large ribosomal subunit. L1 protein does not contain any of these motifs, but it was found to be associated with 5S rRNA. It was suggested that some arginine residues in the protein be involved in RNA binding (Deshmukh et al., 1993). Therefore, it was still possible that Nop2p might be a component of snoRNP by a direct interaction with snoRNAs. Another possibility is that Nop2p might be recruited by snoRNPs through protein-protein interactions.

To investigate the possibility of a protein-protein interaction, immunoprecipitation (IP) was done by using the anti-Nop2p antibodies. In order to increase a chance of detecting the interaction, one monoclonal and one polyclonal antibodies against Nop2p were tested. Since Nop1p is known to be associated with several nucleolar proteins and with at least the 11 yeast snoRNAs (Schimmang et al., 1989), an anti-Nop1p antibody was used as a positive control. [^{35}S] labeled cellular proteins from L4717 cells were precipitated by the antibodies separately, and analyzed with SDS gel electrophoresis. One band at the position corresponding to that of Nop2p was seen in the precipitates with the two anti-Nop2p antibodies, but not with the negative control (without any antibody) (Figure 24A). This suggests that Nop2p was specifically precipitated by the anti-Nop2p antibodies. However, compared with the negative control, no extra band was seen in the precipitates with the two anti-Nop2p antibodies (Figure 24A), suggesting that there was no

Figure 24. Immunoprecipitation analysis of protein-protein and protein-RNA interactions. **(A)** Nop2p is specifically precipitated by the antibodies against Nop2p. L4717 cells in mid-log phase were labeled with [^{35}S]methionine at 25°C for 30 minutes. The whole cell lysate was mixed with the protein-G sepharose-antibody beads at 4°C for 1 hour. A66: monoclonal antibody against Nop1p. D68: monoclonal antibody against Nop2p. APpAb3: polyclonal antibody against Nop2p. Control: protein-G sepharose beads without antibody. The precipitates were separated by SDS-PAGE. The position corresponding to Nop2p is indicated. **(B)** L4717 cells were labeled with ^{32}P-phosphate at 25°C for 30 minutes. The whole cell lysate was used for immunoprecipitation. The precipitated RNAs were extracted and separated by a 7 M urea-6% polyacrylamide gel. **(C)** A nuclear preparation was made from L4717 cells labeled with ^{32}P-phosphate. After immunoprecipitation, the precipitated RNAs were extracted and separated by a 7 M urea-6% polyacrylamide gel.


Nop2p
A
A66
(Anti-Nop1p)
D68
(Anti-Nop2p)
APpAb3
(Anti-Nop2p)
Control
(No Antibody)
B
A66
(Anti-Nop1p)
D68
(Anti-Nop2p)
APpAb3
(Anti-Nop2p)
Control
(No Antibody)
C
A66
(Anti-Nop1p)
D68
(Anti-Nop2p)
APpAb3
(Anti-Nop2p)
Control
(No Antibody)

proteins specifically associated with Nop2p. Considering that the protein-protein interaction might be broken by the *in vitro* treatment, different buffer conditions with a low or high ion concentration were tested. In addition to the whole cell lysate, nuclear homogenate was also used for IP. However, all gave a similar result (not shown). Since in the positive control (anti-Nop1p antibody), many proteins were found in the precipitate (Figure 24A), this indicates that Nop2p was not associated with any proteins under the conditions used here. Nevertheless, it can not be excluded that *in vivo* Nop2p may interact with other proteins through a weak, transient association.

To determine if Nop2p might be associated with snoRNAs, the wild type L4717 cells were labeled with ^{32}P-phosphorus. The whole cell lysate (Figure 24B) and the nuclear preparation (Figure 24C) were used for IP as described above, and the labeled RNAs in the precipitates were extracted and analyzed. Many bands were seen in the precipitate with anti-Nop1p, suggesting that Nop1p was associated with many RNAs (Figure 24B and C). This is consistent with the observations made by other researchers (Schimmang et al., 1989). In the negative control, several bands were also seen, which may be caused by a non-specific binding (Figure 24B and C). Compared with the negative control, no extra band was seen in the precipitates with the two anti-Nop2p antibodies (Figure 24B and C). Considering that no known RNA binding sequence is present in Nop2p, Nop2p may not have a direct association with RNA.

Genetic Approach

Analysis of macromolecule interaction by IP can only reveal those physical interaction that can endure *in vitro* treatment. A weak, transient, but functional interaction can be missed by this approach. The advantage of genetic approach is that it can reveal *in vivo* functional interaction. Reduced affinity caused by mutation in one protein may be complemented by overexpression of another interacting protein. A functional deficit caused

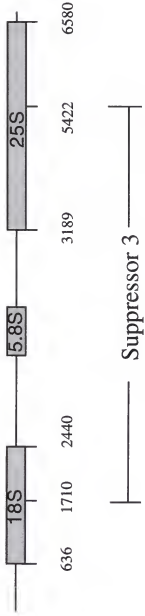
by mutation in one protein may be compensated for by a high level of another protein with homologous function (Huffaker et al., 1987). These are the bases for one genetic method used here, that is, high-copy suppressor screening.

A yeast genomic library carried on a high-copy 2 μm plasmid was transformed into all of the six *nop2* Ts strains. The number of colonies screened for each Ts strain was enough to cover the whole library (Table 12). About 100 plasmids were recovered from the colonies formed at 37°C. Most of the plasmids were different from the library plasmids with a lower molecular weight at ~5-kb, as compared with 6.9-kb of the library plasmid backbone (not including the inserts). The plasmids carrying the *nop2* Ts alleles were also isolated. These *nop2* alleles were present in the strains used for the screening. No *NOP2* gene carried on the library plasmid was isolated. This unexpected result is most possibly due to the way of preparing the library. This library was prepared by *Sau*3A partial digestion of yeast genome (R. Henriquez, personal communication). Since in *NOP2* sequence, there are 10 *Sau*3A sites, it is quite possible that *NOP2* gene might be destroyed during the preparation process. Only one library plasmid with a 3.7-kb insert was isolated because it improved, though very little, the growth of *nop2-10* strain at 37°C (not shown). The subcloned insert exhibited a very weak suppression effect on the phenotype of *nop2-10* strain (not shown). Sequencing of the insert revealed that it was part of rDNA gene, ranging from the middle of 18S to the middle of 25S rRNA sequences (Figure 25). Since the suppression effect was extremely weak and no protein ORF found in the insert, it may not be a specific extragenic suppressor.

Table 12. Screening for High-Copy Suppressors

nop2 allele	Transformants screened	Candidates	Suppressors
nop2-3	5700	4	0
nop2-4	7900	3	0
nop2-5	1600	0	0
nop2-6	2000	50	0
nop2-9	1320	2	0
nop2-10	4900	50	1

Figure 25 . Suppressor candidate 3 contains the part of the rDNA sequence. Suppressor 3, isolated from screening for high-copy suppressors of the *nop2-10* allele, showed only a very weak suppression effect on the temperature-sensitive phenotype caused by *nop2-10*. Sequencing indicates that it contains the part of rDNA, including the 3' half of 18S, 5.8S, and 5' half of 25S rDNA sequences separated by the sequences corresponding to ITS1 and ITS2.


35S
18S
5.8S
25S
ITS 1
ITS 2
636
1710
2440
3189
5422
6580
Suppressor 3

DISCUSSION

Nop2p is Essential for Cell Viability and Growth

Gene disruption in diploid strains, followed by sporulation to test the viability of haploid segregants is thought to be the least biased technique for defining essential genes (Olson, 1991). Previously, it was demonstrated that replacement of 755 bp of *NOP2* ORF with *URA3* caused a lethal phenotype (de Beus et al., 1994). In order to eliminate the possibility of recombination between the chromosome and the complementing plasmid, another gene disruption was made. In this study, over 92% of the *NOP2* ORF in five diploid strains was disrupted and replaced with either a *HIS3* or a *LEU2* marker gene. However, after sporulation and tetrad dissection, all of the four spores from the same ascus were viable with 2:2 segregation for the marker gene. This raised a question about if the gene was essential. However, Nop2p was detected in all of the spores and an intact copy of *NOP2* was present in all of the spores with two of the spores carrying an additional disrupted copy of *NOP2*. This is not a strain-related phenomenon, since five diploid strains were tested with the same result. Apparently, a rare gene duplication event occurred during sporulation stage. During the process of this work, a similar observation was reported (Santos and Ballesta, 1994). When disruption of a gene encoding an essential ribosomal protein, P0, was made in a diploid strain, a possible sporulation-related gene duplication event was also observed (Santos and Ballesta, 1994). Since all of the viable spores carried one copy of *NOP2* gene and expressed it, this phenomenon actually suggests that the gene is required for cell viability. When gene disruption was made in a haploid strain, therefore eliminating the sporulation stage, the viability of the *NOP2*-

disrupted haploid strain was dependent upon the plasmid-carrying *NOP2* gene. This result supports that the gene is essential.

Nop2p function is required for cell growth. During first 8 hours of Nop2p depletion, the cell growth was not affected. This is because the cells contained a high level of Nop2p overexpressed during the pre-growth in YPGal medium. However, further depletion caused a progressive impairment of cell growth. Over 90% of the cells depleted of Nop2p for 28 hours were still viable. This is probably due to a basal activity of *GAL10* promoter even in the presence of glucose repressor, a phenomenon also observed in the other depletion studies (Moritz et al., 1990; Deshmukh et al., 1993). Stronger evidence is from the mutational analysis of *NOP2* gene. All of the six Ts strains containing the mutant protein were dead when incubated at 37°C, but the lethal phenotype was rescued by a plasmid-borne *NOP2* gene. These results confirm that *NOP2* gene is indispensable for cell viability and growth.

Nop2p is Required for Ribosome Synthesis

The fact that *NOP2* gene is essential for cell viability and growth indicates its important cellular functions. Depletion of Nop2p caused a significant reduction of the 60S ribosomal subunit but a relative accumulation of the 40S subunit. A relatively specific feature associated with the shortage of 60S subunit is the halfmer polysome, which was observed during depletion of the large subunit protein L1 or L16, and during depletion of Nop4p/Nop77p, a nucleolar protein involved in the synthesis of 60S subunit (Moritz et al., 1990; Deshmukh et al., 1993; Berges et al., 1994). The cells depleted of Nop2p clearly exhibited this feature. Since the reduction of 60S subunit level was observed before the cell growth slowed down, the defect was directly caused by Nop2p depletion.

A more convincing evidence is from the mutational analysis of the protein. All of the mutant protein generated at the restrictive condition will be almost immediately

inactivated, therefore eliminating possible secondary effects. In all of the six *nop2* Ts strains, two hours of incubation at 37°C caused a significant defect in the synthesis of 60S subunit. Some of the Ts strains exhibited an abnormal polysome profile even under permissive conditions, suggesting that the severe mutations affected the protein conformation even at the permissive condition. In the *nop2-10* strain, pre-rRNA processing at 25°C is similar to that in the control strain. However, at this temperature, a defect in the synthesis of the 60S ribosomal subunit was obvious, which suggests that Nop2p may be involved in the other steps of ribosome synthesis as well as in pre-rRNA processing. The results from the depletion and mutation studies of Nop2p clearly indicate that Nop2p is essential for synthesis of 60S ribosomal subunit.

Synthesis of ribosome involves multiple steps, including transcription of rDNA gene, processing and modification of pre-rRNA, expression of ribosomal proteins, assembly of rRNAs with ribosomal proteins, and export of ribosomal subunits to the cytoplasm.

Nop2p is Required for Pre-rRNA Processing

rDNA is transcribed as a single large pre-rRNA, which contains the sequences of rRNAs. Processing of the pre-rRNA into mature rRNAs is an important event in ribosome synthesis. Since a general outline of processing of pre-rRNA has been known, pulse labeling of pre-rRNA and chasing it to mature rRNAs will reveal a defect in the processing. The processing of pre-rRNA in the wild type L4717 was finished within 5 minutes, as was expected (Girard et al., 1992; Russell and Tollervey, 1992). Depletion of, and mutation in Nop2p caused a significant reduction in the formation of 25S rRNA, but the processing to 18S rRNA was not significantly affected. This suggests that Nop2p is involved in the step after the separation of 18S and 25S pathways. Analysis of the accumulated processing intermediates revealed a significant accumulation of 27S intermediate in Nop2p-depleted

cells and in several of the Ts strains (*nop2-3*, *nop2-6*, *nop2-9*, and *nop2-10*). Analysis of the steady-state levels of the processing intermediates indicates that both 27SA and 27SB intermediates significantly accumulated in the Nop2p-depleted cells. This suggests that the defect caused by the deficit in Nop2p function was at the step of conversion of 27SB into 25S rRNA and 7S, a direct precursor of 5.8S rRNA. Reduction in 5.8S rRNA during Nop2p depletion supports this conclusion. Based on these results, we conclude that Nop2p is required for the processing in ITS2, probably at C_2 site (see Figure 10).

Several yeast nucleolar proteins have also been shown to be required for the formation 25S rRNA. However, depletion of them separately exhibited different features from depletion of Nop2p, and they are required for processing at the sites different from the one requiring Nop2p. Nop4p/Nop77p was found to be involved in the processing pathway to 25S rRNA. However, depletion of Nop4p/Nop77p caused a significant reduction in the 27SB intermediate, which suggests that it may function at the 27SA level within 3' half of ITS1 (Berges et al., 1994; Sun and Woolford, 1994). Nop3p, though localized in the nucleus, was also found to be required for the processing of 27SB to 25S rRNA (Russell and Tollervey, 1992). However, this protein is also involved in the processing of 20S intermediate to 18S rRNA, the only processing step occurring in the cytoplasm. Depletion of Nop3p for a long period of time revealed the accumulation of both 23S and 27SB processing products (Russell and Tollervey, 1992), which arise from cleavage of the 35S primary transcript at or near site B_1 without prior cleavages at A_1 and A_2 sites. This suggests that Nop3p is possibly required for the processing in ITS1. Dbp3p is required for the processing at A_3 site in ITS1. Depletion of Dbp3p caused an accumulation of 27SA but a reduction of 27SB intermediate (Weaver et al., 1997). In the yeast *rrp1* Ts mutant, the 27S intermediate was rapidly degraded (Fabian and Hopper, 1987). Since ribosome assembly and processing of pre-rRNA occurs simultaneously, a defect in the assembly may affect the processing. Temperature-sensitive mutations in L16, a protein component of 60S subunit, caused a defect in the processing of 27SB to 25S

rRNA. However, the 27SB and other processing intermediates were rapidly degraded (Moritz et al., 1991). Therefore, Nop2p is probably the first of the nucleolar proteins identified so far, which is required for processing in ITS2. Disappearance of the 27S intermediate in *nop2-4* and *nop2-5* strains is most possibly caused by a rapid degradation, since primer extension analysis of nop2-4 strain incubated at 37°C for two hours did not detect any change in the methylation of the 27S sequence. These results suggest that Nop2p is involved in the stability of the 27S intermediate.

Possible Roles of Nop2p in the Other Events of Ribosome Synthesis

Nop2p and rDNA transcription. The role of Nop2p in the transcription of rDNA gene was evaluated by pulse-chase labeling with [^{3}H]uracil. In all of the six Ts strains and the cells depleted of Nop2p, a significant accumulation of 35S transcript, which is the largest recognizable precursor for rRNAs (Woolford and Warner, 1991), was observed, indicating that rDNA was still transcribed in Nop2p-depleted cells or in the *nop2* Ts cells. This suggests that Nop2p is not involved in the transcription of rDNA gene.

Nop2p is not a structural component of 60S ribosome subunit. Depletion of a protein component of 60S subunit will also reduce the level of the subunit and cause a rapid degradation of 25S rRNA and the other components of this subunit. Since Nop2p is mainly localized in the nucleolus (de Beus et al., 1994), it is not possibly a structural component of 60S ribosomal subunit. Depletion or mutation of Nop2p caused not only a reduction in 25S rRNA but also an accumulation of 35S 32S and 27S pre-rRNAs, suggesting that there was a delay in the pre-rRNA processing, and that the reduction of 25S rRNA was not caused by a rapid degradation due to disruption of 60S subunit.

Nop2p and ribosome assembly. In prokaryotes, ribosome subunits can be assembled *in vitro* (Nomura, 1973). However, this has not be successful in eukaryotes at

the current time, which reflects the complication and multiple factors required in eukaryotes. Therefore, currently, it is difficult to analyze the direct role of a nucleolar protein in the assembly process. Although Nop2p does not contain a GAR domain or an acidic domain, it can not be excluded that the acidic or basic residues in the protein sequence may facilitate ribosome assembly. It is also possible that Nop2p may modify ribosomal protein(s) or rRNAs, and therefore promotes the correct assembly of ribosomes.

Nop2p and nucleolus-cytoplasmic transport. Nucleolin and B23 were found to shuttle between the nucleolus and the cytoplasm (Borer et al., 1989), suggesting that they may be involved in the transport between these two compartments. There is no direct evidence suggesting that Nop2p has such a function. Since a small portion of Nop2p was found to be in the cytoplasm (de Beus et al., 1994), it is possible that Nop2p may also shuttle between the nucleolus and the cytoplasm to help transport of the ribosomal components or the mature ribosome.

Interaction of Nop2p with Other Macromolecules

Intracellular macromolecules related to the same functional pathway usually interact physically and/or functionally with each other. Analysis of such interactions will help understand more about the protein function. Several approaches have been used in this study in order to detect any functional or physical association of Nop2p with other macromolecules. Immunoprecipitation was successfully used for analysis of association of Nop1p, Gar1p or Sof1p with snoRNAs and nucleolar proteins (Schimmang et al., 1989; Girard et al., 1992; Jansen et al., 1993). However, no such association was found with Nop2p.

Since Nop2p does not contain any of the known RNA binding motif, such as RRM or GAR domain, it may not directly associate with any RNAs. However, a weak protein-protein association between Nop2p and other proteins may still exist. Such association

may not survive the *in vitro* treatment. One piece of evidence supporting this assumption is from the mutational analysis of Nop2p. In *nop2-4* and *nop2-5* strains incubated at 37°C, the 27S pre-rRNA was rapidly degraded, while in the other Ts stains and Nop2p-depleted cells this pre-rRNA significantly accumulated. This suggests that there may be a region in Nop2p that is related to the association of other nucleolar proteins with 27S pre-rRNA, and that the mutations in *nop2-4* and *nop2-5* alleles disrupt such association, resulting in a rapid degradation of this pre-rRNA. The yeast two hybrid system can detect intracellular protein-protein interaction, and therefore is a more sensitive method than immunoprecipitation. Nevertheless, no protein interacting with Nop2p was isolated (Wu, P. and J. P. Aris, unpublished results). The search for high-copy suppressors of the *nop2* alleles also gave a disappointing result. Although there have been several successful application of this genetic approach, the limitation is that suppressors isolated may not be functionally related to the protein of interest (bypass suppressors) because it aims for "gain of function" (Doye and Hurt, 1995). In this study, a piece of rDNA sequence isolated may belong to the "bypass suppressors". Another genetic approach, synthetic lethal screen, is based on "loss of function", and has been extremely successful (Doye and Hurt, 1995). Screening for the genes synthetic lethal with *NOP2* alleles is currently under way in this lab.

The Mechanism by Which Nop2p Functions

Almost all of the yeast nucleolar proteins identified so far have been shown to be involved in processing of pre-rRNA. Only a few of them, such as Rnt1p (RNase III), Xrn1p and Rat1p, were found to have direct nucleolytic activities (Stevens et al., 1991; Amberg et al., 1992; Elela et al., 1996). However, the molecular mechanisms for the functions of the other nucleolar proteins in pre-rRNA processing have not been clearly understood. Many nucleolar proteins contain a RNA binding motif, such as RRM or GAR domain. It can be imagined that some nucleolar proteins may bind to pre-rRNA to facilitate

its folding, which is important for the processing. Since some nucleolar proteins were shown to be physically and functionally associated with snoRNAs, they may function as components of a snoRNP complex. One of the features about Nop2p is that there is no RRM or GAR domain present in its sequence (Aris, J. P. and B. Hong, unpublished results). Therefore, the underlying molecular mechanism for Nop2p may be different from those for the other nucleolar proteins.

Nop2p and rRNA Methylation

Proteins with their functions essential for viability are usually evolutionarily conserved. Comparison of protein sequences will reveal conserved motifs and offer a clue to the protein function. Protein sequence alignments indicates that Nop2p contains the SAM binding domain, which is conserved in methyltransferases. Since rRNAs are greatly modified with methylation, this raises an interesting possibility that Nop2p may have a rRNA methylase activity. During Nop2p depletion, the methylation at the $UmGm\Psi UC_{2922}$ site in 25S rRNA sequence was reduced. The methylation at this site has been mapped at the 27S intermediate level (Brand et al., 1977), and processing of this intermediate to 25S rRNA may require Nop2p function. Therefore, the methylation and the processing is clearly related. Since this stretch of sequence and the methylation sites are evolutionarily conserved from bacteria to human, it is very possible that the methylation at this site is essential for ribosome synthesis and ribosome function. Methylation increases local hydrophobicity, and therefore may help to form the secondary structure of pre-rRNA, which may be required for the processing or for the binding of ribosomal proteins to 27S intermediate.

If this is true, then the methylation is required for the processing. Such relationship was observed in the cultured mammalian cells. When deprived of methionine or treated with an inhibitor of methyl transfer, the cells had a defect in the processing of pre-rRNA

(Swann et al., 1975; Wolf and Schlessinger, 1977). However, to prove the causal relationship, it has to be confirmed that the methylation occurs before or at the same time as the processing. Technically, it is difficult to address this issue *in vivo*. Another approach can be used for this purpose is to make a point mutation in rDNA corresponding to this methylation site so that the methylation can not occur. It will be a very convincing evidence if a defect in the processing of the mutated 27S intermediate still occurs.

However, methylation may be only temporally related to processing, but not required for processing. In yeast *nop1-3* mutant, the demethylated pre-rRNA was still processed (Tollervey et al., 1993). Yeast Dim1p, an 18S rRNA methylase, modifies a conserved site on 18S rRNA. Although the protein is essential, the methylation is not required for the processing (Lafontaine et al., 1995). Since there are 65 methylation sites on yeast rRNAs, it is possible that some of the methylations may be required for the processing of pre-rRNA and for the function of mature ribosome, while the others may be not essential.

To determine if Nop2p might be directly involved in methylation, an *in vitro* methylation reaction was performed in this study. The possibility of direct catalysis of [*methyl* -^{3}H] transfer to an 27S *in vitro* transcript by Nop2p was investigated. The 27S transcript and Nop2p purified by immunoprecipitation were incubated in the buffer containing [^{3}H]-S-adenosyl-methionine (see Methods and Materials) at 30°C for 1 hour. A 3.8-kb band corresponding to the 27S transcript was clearly visible on the gel stained with ethidium bromide. However, compared with the control reaction (without adding Nop2p), no isotope incorporation or any other change in the band pattern was seen (not shown). Since no positive control was included in this experiment, it is difficult to evaluate if the reaction conditions were appropriate for methylase activity. In the future, if available, a positive control, such as Dim1p (a yeast 18S methylase), should be included in the methylation reaction. The involvement of Nop2p in methylation could not be detected by the primer extension assay. This is most possibly due to the limitation of the method.

However, it also suggests that there may be other possible mechanisms by which Nop2p functions in pre-rRNA processing.

Nop2p and Protein Methylation

Another possibility is that Nop2p may be a protein methylase. One piece of evidence to support this assumption is from the observation that a protein(s) seemed to be demethylated during Nop2p depletion, though the result is still inconclusive (Brokenbrough, J. S. and J. P. Aris, unpublished results). Based on the modified amino acid residues, protein methyltranferases can be classified into several groups. One group, protein methylase I, recognizes and modifies the arginine residues in the GAR domain (Lischwe, 1990; Paik and Kim, 1990). Nucleolin, fibrillarin and yeast Ssb1p have been shown to be methylated on the arginine residues, though the function of, the enzymes responsible for, and the cellular location of, such modification have not been understood (Lischwe, 1990). Since there is no known RNA binding motif present in Nop2p sequence, and no association between Nop2p and RNA has been found, it is quite possible that Nop2p may not directly modify RNA. One scenario is that Nop2p may modify a protein with RNA methylase activity. The modified protein then transfers methyl groups to the $UmGm\Psi UC_{2922}$ site. Or this site may be modified only after the cleavage at the C_2 site in ITS2 has been completed. Nop2p may modify a protein component of a snoRNP complex, which is required for the processing at the C_2 site. With such modification, the snoRNP may assume a functional conformation, bind to the 27S intermediate and cleave it at the C_2 site. Since snoRNPs have been proposed to be involved in a variety of the events in ribosome synthesis, including modification and processing of rRNA, methylation of protein components in an snoRNP may be one of the mechanisms by which Nop2p functions in pre-rRNA processing.

Nop2p and RNA Helicase

ATP-dependent RNA helicases containing the conserved DEAD-box have been shown to be required for pre-rRNA processing, possibly by facilitating the formation of secondary structure of pre-rRNA. Drs1p, Dbp3p, and Spb4p are all involved in the processing 27S intermediate to 25S rRNA (Sachs and Davis, 1990; Ripmaster et al., 1992; Weaver et al., 1997). A partial DEAD sequence is present in Nop2p (J. P. Aris, unpublished results). However, it is not known if the DEAD sequence in Nop2p plays any role in pre-rRNA processing. Since there is no evidence to support that Nop2p is associated with any RNA, it is unlikely that Nop2p will be directly involved in folding of pre-rRNA.

Nop2p and SnoRNP

Since Nop2p is involved in both rRNA methylation and the cleavage at C_2 site in ITS2, it is possible that Nop2p may have multiple functions. One part of the protein with the SAM binding motif as the activity center may function as a rRNA or protein methylase. Another part may be involved in the cleavage. Nop2p is possibly not a nuclease, since no cleavage of a 27S rDNA transcript by Nop2p was observed *in vitro*, though the possibility can not be excluded that the transcript may not form the correct secondary structure. Nop2p may not be directly associated with any RNA. One possibility is that the part of the protein may be involved in association with a snoRNP through a weak protein-protein interaction. Such association may be required for the binding of the snoRNP to 27S intermediate, and therefore the nucleolytic activity of the snoRNP. Depletion of Nop2p made the protein less available for such association, resulting in a defect in cleavage at C_2 site. In *nop2-4* and *nop2-5* strains but not in the other Ts strains, 27S intermediate was rapidly degraded at the restrictive condition, suggesting that the conformational change of

the mutant protein may affect the binding of the snoRNP to 27S intermediate. The 27S intermediate without the binding of the snoRNP may become less stable and rapidly degraded.

In conclusion, depletion and mutation of Nop2p indicate that Nop2p is required for synthesis of the 60S ribosomal subunit and processing of pre-rRNA to 25S and 5.8S rRNAs. RNase protection suggests that Nop2p is involved in the methylation at the $UmGm\Psi UC_{2922}$ site of 25S rRNA sequence. Sequence alignment reveals that Nop2p contains a SAM-binding domain. These results indicate that Nop2p may have methyltransferase activity. Since there is no RNA binding domain or motif present in the Nop2p sequence and since no RNA was found to be associated with Nop2p in immunoprecipitation assay, Nop2p may modify a protein component of a snoRNP complex. The modified snoRNP complex may then modify and process pre-rRNA. Through characterizing Nop2p function, this study helps to illustrate the molecular mechanisms by which nucleolar proteins function in modification and processing of pre-rRNA. Nop2p is an essential nucleolar protein with a significant sequence homology to human nucleolar protein p120 (de Beus et al., 1994). Therefore, this study will also help reveal the role of human p120 protein in normal and tumor cells.

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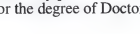

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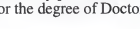

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